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CORRIGENDUM

The isolation and characterization of acetylcholine-containing particles from brain

By V. P. WHITTAKER

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Steps in the Oxidation of Xanthine to Uric Acid Catalysed by Milk Xanthine Oxidase

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The development of various rapid-reaction techniques to study a number of distinct steps involved in individual enzyme catalysed reactions has been very successful in application to haem enzymes (Chance, 1951), diphosphopyridine nucleotide-linked systems (Theorell & Chance, 1951) and hydrolytic enzymes (Gutfreund, 1955). Speculations and preliminary evidence for the existence of intermediates in flavoprotein systems (Beinert, 1957) have encouraged us to study one of these in detail by fast-reaction spectroscopy. Milk xanthine oxidase has recently been crystallized by Avis, Bergel & Bray (1955), and it was found by Avis, Bergel, Bray, James & Shooter (1956) that the enzyme has a molecular weight of approximately 300 000, that it contains 2 flavinadenine dinucleotide groups, 8 iron atoms and 1–2 molybdenum atoms/molecule. Previous detailed studies of the catalytic activity (Morell, 1952; Mackler, Mahler & Green, 1954) made this enzyme system a most suitable one for a first attempt to apply new methods to the study of flavoprotein-catalysed reactions. The studies reported here can be regarded as an extension of Morell's investigations to the kinetics of the anaerobic reduction of the enzymic flavinadenine dinucleotide by xanthine, to the reoxidation of substrate-reduced enzyme by molecular oxygen, and of the steady-state formation of uric acid catalysed by the enzyme in the presence of xanthine and oxygen. Furthermore, the kinetics of the reduction and reoxidation of the enzymic flavinadenine dinucleotide was followed at different wavelengths between 400 and 600 mμ in a search for spectroscopically observable intermediates in these reactions. From the results of these experiments a complete kinetic scheme can be written for the enzymic conversion of xanthine into uric acid and in conjunction with data reported by others, preliminary suggestions for a mechanism can be proposed for this flavoprotein-catalysed hydrogen transfer.

EXPERIMENTAL

Methods. All spectra and activity measurements were obtained by use of a Unicam Instrument Co. Ltd. (Cambridge) SP. 500 spectrophotometer. The rapid reaction measurements were started for exploratory purposes in a Gibson (1952) stopped-flow apparatus, slightly modified for enzyme work (Gutfreund, 1955). All the quantitative kinetic measurements were carried out in a stopped-flow attachment to the Unicam spectrophotometer described by Spencer & Sturtevant (1959). This was kept at 25°. The observation cell of the stopped-flow mixing device has a 1 cm. light-path and fits in the place of the usual cell-holder. The optical system and photocell of the Unicam instrument were used for measurements of extinction under static conditions, while only the monochromator of the Unicam instrument and a separately attached (Spencer & Sturtevant, 1959) photomultiplier, amplifier and Bush Electronic Co. (Cleveland, Ohio) Model BL-201 pen-and-ink recorder were used for recording rapid changes during the course of enzymic reactions. This latter arrangement gives a record of percentage transmission which is converted into change in extinction for the calculation of rate constants.

Anaerobic experiments were carried out with solutions which had been shaken up *in vacuo* and then under nitrogen, this procedure being repeated three times before transfer of the solutions under anaerobic conditions into the syringes of the fast-reaction apparatus. Experiments at different concentrations of oxygen were carried out with solutions which had been treated *in vacuo* and had then been equilibrated with appropriate pressures of oxygen at 25°. The molarity of oxygen was calculated from the oxygen pressure on the basis that the solubility of oxygen in water at 25° at 1 atm. of O_2 is 1·23 mM.

Materials. Xanthine was obtained from Roche Products Ltd. and solutions of required concentration were made up in 0·1 M-sodium pyrophosphate adjusted to pH 8 with HCl with a glass electrode.

All xanthine oxidase preparations used were obtained from Professor F. Bergel, Dr R. C. Bray and Mr D. A. Gilbert of the Chester Beatty Research Institute and had been prepared from cow's milk by the method of Avis *et al.* (1955). Various individual pilot experiments were carried out with small special samples of the enzyme, but all the fast-reaction measurements from which quantitative conclusions are drawn in this paper were carried out with one preparation. The enzyme was obtained in solutions which contained 0·8 M-sodium phosphate (pH 7·5) and 4 mg. of sodium salicylate/ml. and it retained constant activity if stored at 2° for several months. The principal sample of xanthine oxidase was at stage m6 (before crystallization) in the scheme of purification of Avis *et al.* (1955). The protein concentration of enzyme solutions was not measured, but the concentration of total flavinadenine dinucleotide (FAD) and enzymically active FAD was estimated from the $E_{450\ m\mu}$ of the solution and from the change in $E_{450\ m\mu}$ on anaerobic reduction by xanthine. The method of interpretation of the extinction data was based on

Morell's (1952) results. The extinction, $E^{1\,cm.}_{450\,m\mu}$, of the stock solution was 1·22. The amount of enzyme required for a day's experiments was dialysed overnight against 0·1 M-sodium pyrophosphate, pH 8·0. After dialysis and slight dilution during washing of the dialysis sack, the extinction was reduced to approximately 0·85. In the mixing chamber the enzyme concentration was halved; all concentrations and extinctions quoted in the descriptions of kinetic experiments are those of the reaction mixtures after mixing enzyme and substrate solutions. Precise data for the extinctions of reaction mixtures are given in appropriate sections of the paper.

RESULTS

The spectrum of the xanthine oxidase stock solution, as well as the difference spectrum between oxidized and reduced enzyme, were very similar to the figures shown by Morell (1952). We have found, in agreement with Morell (1952), that the specific enzyme activity of different samples of xanthine oxidase is proportional to the percentage reduction of $E_{450\,m\mu}$ on anaerobic addition of xanthine. Using Morell's method of calculating the percentage of enzymically active FAD, we found that this corresponded in our stock sample to 33 % of the total flavine present. Like Morell we found that anaerobic reduction by xanthine was biphasic, consisting of a rapid reduction to 33 % of the total reduction by sodium dithionite, and a subsequent, very slow reduction which we interpreted, following Morell, as the reduction of FAD bound to inactive enzyme. First, we investigated in some detail the kinetics of the anaerobic reduction of xanthine oxidase by sodium dithionite and by xanthine.

Reduction of xanthine oxidase by sodium dithionite. The reduction of the enzyme by sodium dithionite was followed anaerobically by observing the change in $E_{450\,m\mu}$. The reaction mixture contained, apart from xanthine oxidase, 0·05 M-sodium dithionite and 0·1 M-sodium pyrophosphate, pH 8·0. The initial $E_{450\,m\mu}$ was 0·457 and the final value 0·179. Using a simple plot of log $(E-E_t)$, which is referred to below as log ΔE, against t, we found that the reaction was pseudo-first-order through 70 % of its course ($k = 0{\cdot}12$ sec.$^{-1}$).

Anaerobic reduction of xanthine oxidase by xanthine. The rapid decrease in extinction on mixing oxygen-free solutions of xanthine oxidase and xanthine was observed at 400, 450, 500 and 550 mμ. Only at 450 mμ was the change in extinction sufficiently large to make accurate calculations of the time course of the reduction possible. From observations at the other wavelengths one could only draw the conclusion that the half-life of the reaction described is the same. Fig. 1 *A* shows the change in $E_{450\,m\mu}$ calculated from the record of such an experiment. The slow drift following the rapid change corresponds to the slow reduction of enzymically inactive FAD, and this was corrected for before calculation of the rate constant of the rapid reduction of the enzyme. As can be seen from the plot of log ΔE against t recorded in Fig. 2, this reaction follows first-order kinetics ($k = 10{\cdot}4$ sec.$^{-1}$). A number of experiments showed that for substrate concentrations ranging from 500 to 50 μM the rate of the anaerobic reduction is characterized by $k = 10{\cdot}5 \pm 1{\cdot}0$ (max. deviation) sec.$^{-1}$

Reduction of xanthine oxidase by xanthine in the presence of oxygen. When the course of the reduction of xanthine oxidase was followed after mixing enzyme and xanthine in the presence of oxygen, it was found that $E_{450\,m\mu}$ was decreased by approximately one-third of the anaerobic reduction caused by xanthine and that it remained at this level throughout a steady-state period. At the end of the

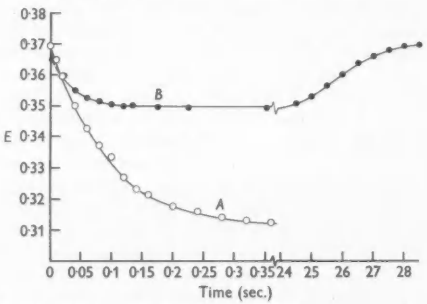


Fig. 1. *A*, Anaerobic reduction of xanthine oxidase ($E^{1\,cm.}_{450\,m\mu}$ 0·37) by xanthine (0·25 mM) in 0·1 M-sodium pyrophosphate, pH 8·0. The reactions were followed by recording the percentage transmission at 450 mμ and the data were converted in E units. *B*, Reduction of xanthine oxidase ($E^{1\,cm.}_{450\,m\mu}$ 0·37) by xanthine (0·25 mM) in the presence of oxygen (0·6 mM). (The concentrations are those after mixing of enzyme and substrate solutions.)

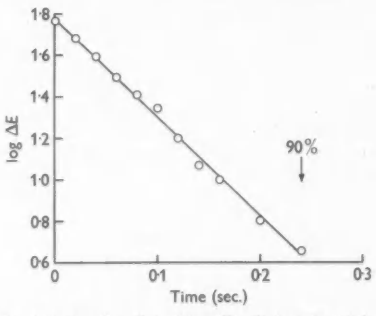


Fig. 2. A first-orderp lot of log $(E-E_\infty)$ against t for the experiment in Fig. 1 *A*.

reaction the extinction either dropped to the xanthine-reduced level, if the concentration of xanthine was greater than that of oxygen, or rose to the fully oxidized level, if oxygen was in excess of xanthine. Fig. 1 shows the record of two experiments, one with oxygen-free solutions and the other containing 0·6 mM-oxygen in the reaction mixture, the xanthine concentration being 0·25 mM in both reaction mixtures. We studied the rate of reduction of the enzyme to the steady-state level over a range of initial oxygen concentrations from 1 to 0·1 mM. From six measurements of the rate of reduction to the steady state, at initial oxygen concentrations greater than 0·2 mM we calculated a first-order rate constant 32 ± 2 (max. deviation) sec.$^{-1}$. At lower concentrations of oxygen this rate constant decreased, but since under those conditions no steady-state period is reached it becomes too complicated to evaluate individual rate constants. Approximate values for the half-optimum concentration of oxygen were obtained from measurements of the overall velocity of the enzyme reaction as described in the next section.

Rate of appearance of uric acid and the Michaelis parameters for the overall reaction. The kinetics of uric acid formation under steady-state conditions can be calculated from observations of the change in $E_{310\,m\mu}$. We used this wavelength, at which the change of ϵ on oxidation of xanthine to uric acid is $2{\cdot}1 \times 10^3$ l. M^{-1} cm.$^{-1}$, rather than 290 mμ, where the change in ϵ of this reaction is at its maximum, $9{\cdot}6 \times 10^2$ l. M^{-1} cm.$^{-1}$ (Kalckar, 1947). The observation of the rate of formation of uric acid under identical conditions of enzyme and substrate concentrations as used in our measurements of the reduction and reoxidation of the enzyme involves the presence of relatively large amounts of enzyme and products during the course of the experiment, and the extinction at 290 mμ became too great, while at 310 mμ satisfactory observations could be made and Beer's law was obeyed.

Fig. 3*A* gives the plot of $E_{310\,m\mu}$ against time for a typical experiment. The initial substrate and oxygen concentrations in the reaction mixture were 0·25 and 0·6 mM respectively. Fig. 3*B* is from a similar experiment in 50 % (v/v) ^2H_2O, the results of which are discussed below. The reaction was zero-order through approximately 80 % of its course and the xanthine concentration at half-optimum velocity was estimated to be 20 μM. This is not regarded as an accurate value for the Michaelis constant, since measurements in the fast-reaction apparatus are not suitable for the precise evaluation of this constant; it is, however, in good agreement with the detailed kinetic investigation of Hofstee (1955), who obtained $K_m = 20\,\mu$M.

From the record of the appearance of uric acid in one experiment at a low initial concentration of oxygen we have estimated an approximate value for half-optimum oxygen concentration. Fig. 4 shows the rate of appearance of uric acid for an experiment at initial xanthine and oxygen concentrations of 0·25 and 0·09 mM respectively. The scale on the left-hand side gives the change in extinction due to the appearance of uric acid and the scale on the right-hand side gives the calculated oxygen concentration. The dotted line gives the calculated limiting velocity at high oxygen concentration for

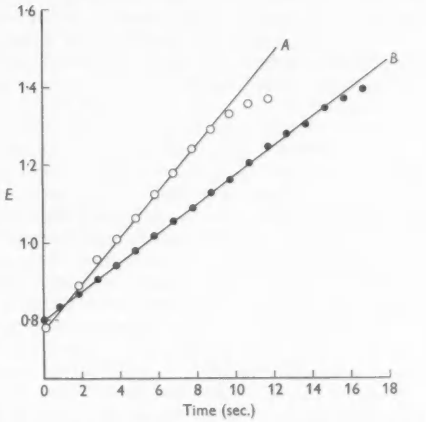


Fig. 3. Change in $E_{310\,m\mu}$ during the xanthine oxidase-catalysed formation of uric acid from xanthine in 0·1 M-sodium pyrophosphate, pH 8; initial concentrations of xanthine and oxygen 0·25 and 0·6 mM respectively; xanthine oxidase, $E^{1\,cm.}_{450\,m\mu}$ 0·40. Curve *A* is from an experiment in aqueous solution, curve *B* from an experiment in 50 % (v/v) ^2H_2O. (The concentrations are those after mixing of enzyme and substrate solutions.)

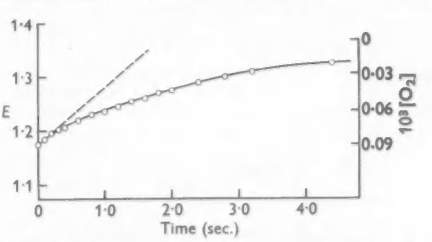


Fig. 4. Change in $E_{310\,m\mu}$ during the xanthine oxidase-catalysed formation of uric acid from xanthine in 0·1 M-sodium pyrophosphate, pH 8·0; initial concentrations of xanthine and oxygen 0·25 and 0·09 mM respectively; xanthine oxidase, $E^{1\,cm.}_{450\,m\mu}$ 0·251. The broken line gives the calculated initial velocity at optimum oxygen concentration. (The concentrations are those after mixing of enzyme and substrate solutions.)

the enzyme concentration ($E_{450\,m\mu}$ 0·388) and the half-optimum concentration can be estimated as 50 μM.

The overall rate constant of the enzyme for optimum concentrations of xanthine and oxygen has been calculated from the molarity of active enzyme and the rate of appearance of uric acid. The method for this calculation is described in detail by Morell (1952). The assumptions involved in the calculations of the molarity of active enzyme FAD sites from the molar extinction coefficient of enzymic FAD, the percentage reduction and the initial $E_{450\,m\mu}$ of the enzyme solution make estimates of moles of uric acid formed/mole of enzyme/sec. rather inaccurate. The value of 8 ($\pm$ 0·5) sec.$^{-1}$ represents the range of values calculated from six experiments.

Effect of D_2O *on different steps of the hydrogen-transport process.* Both the rate of anaerobic reduction of xanthine oxidase by xanthine and the overall rate of the steady-state formation of uric acid in the presence of oxygen have been determined in solutions which contained 50 % (v/v) of ^2H_2O. The enzyme solution was made up in water as above, but the substrate was dissolved in D_2O which contained phosphate equivalent to that in the aqueous buffers used. As can be seen in Fig. 5, the two comparative kinetic measurements of the rate of anaerobic reduction at identical substrate concentration, $[S_0]$ = 0·25 mM, in H_2O and 50 % (v/v) H_2O–^2H_2O mixtures respectively gave indistinguishable results (k = 10·5 sec.$^{-1}$).

Measurements of the rate of appearance of uric acid were carried out with solutions of the same concentrations of xanthine oxidase and xanthine as in the above anaerobic experiments but containing in addition 0·6 mM-oxygen. Two parallel experiments under identical conditions, except that in one reaction-mixture H_2O was the solvent while in the other a 50 % (v/v) mixture of H_2O and ^2H_2O was the solvent, are recorded in Fig. 3. The rate of the overall enzyme reaction in 50 % ^2H_2O is only 64 % that of the rate in H_2O.

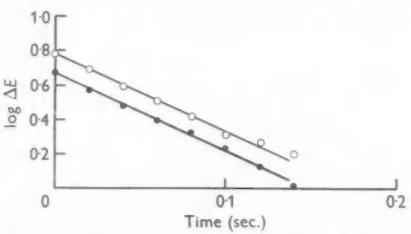
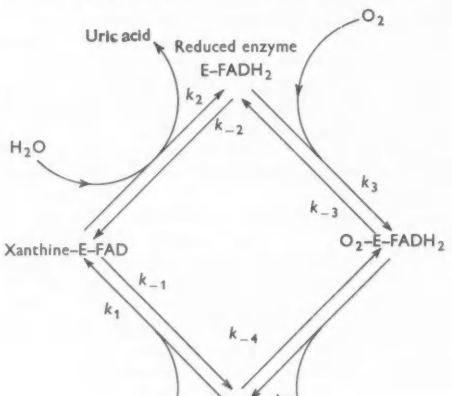


Fig. 5. Comparison of first-order plot of rate of anaerobic reduction of xanthine oxidase by xanthine in water (○) and in 50 % (v/v) ^2H_2O (●); 0·1 M-sodium pyrophosphate, pH 8·0; initial xanthine concentration, 0·25 mM; xanthine oxidase, $E^{1\,cm.}_{450\,m\mu}$ 0·251. (The concentrations are those after mixing of enzyme and substrate solutions.)

DISCUSSION

The experiments with xanthine oxidase reported here were carried out primarily to explore the potentialities of spectroscopic fast-reaction techniques in the analysis of individual steps of hydrogen transfer catalysed by a readily available flavoprotein. We have concentrated on those features of the enzyme reaction which are common to all flavoproteins. We have neglected the possible role of metals, which has received very wide attention recently (Nicholas, 1957; Bergel & Bray, 1958), because many flavoproteins can participate in similar hydrogen-transport processes without the help of metals. It is our intention to use the experience gained here to select and study much simpler flavoproteins in greater detail.

Our most important conclusions are that both xanthine and oxygen combine rapidly with enzyme to form Michaelis–Menten compounds, before the reduction and reoxidation of the FAD moiety of the enzyme and that the steady-state ratio of $FADH_2$ (reduced flavinadenine dinucleotide) to FAD during turnover of the enzyme at optimum concentrations of xanthine and oxygen corresponds to the ratio of the first-order rate constants for reduction and reoxidation. To arrive at these conclusions we had to determine a sufficient number of the rate constants of the cyclic reaction (Scheme 1).



Scheme 1

When we studied the anaerobic reduction of xanthine oxidase by xanthine we found that the reduction of FAD, as observed by changes in $E_{450\,m\mu}$, followed first-order kinetics and was independent of xanthine concentration over a wide range. We concluded from this that the value of 10·5 sec.$^{-1}$ found as rate constant for the reduction process can be assigned to the step characterized by k_2; an initial second-order xanthine–enzyme combination, characterized by k_1, must precede this step. From our own and Hofstee's (1955) data we estimate the xanthine K_m to be 20 μM; since $K_m = (k_2 + k_{-1})/k_1$ one can give a minimum value for k_1 of 50 μM^{-1} sec.$^{-1}$.

Our findings, that the reduction of enzymic FAD to the steady-state level in the presence of oxygen is also a first-order process and is characterized by a rate constant three times that of k_2, can be explained as follows. Since the steady-state ratio of oxidized to reduced enzyme is independent of the oxygen concentration above 0·5 mM, one can conclude that oxygen too forms a compound with the enzyme before its oxidation of the enzymic FAD. From the dependence of the rate of the overall appearance of uric acid on the concentration of oxygen an estimation of the oxygen K_m (50 μM) was made and k_3 (> 40 μM^{-1} sec.$^{-1}$) can be calculated in a manner analogous to the calculation of k_1 above. If one accepts that the second-order combination of enzyme with both xanthine and oxygen is rapid compared with the first-order reduction and reoxidation of the enzymic FAD, then one can regard the steady-state level as an equilibrium between two opposing reactions of the xanthine–enzyme compound with the oxygen–reduced-enzyme compound

$$\text{Xanthine–E–FAD} \underset{k_4}{\overset{k_2}{\rightleftharpoons}} \text{O}_2\text{–E–FADH}_2.$$

The rate constant of the reaction to the equilibrium position of two first-order opposing reactions, k_s, is equal to $k_2 + k_4$ and is itself a first-order constant. We have obtained a value for k_s of 32 sec.$^{-1}$ from our experiments on the reduction of enzyme by xanthine in the presence of oxygen, and from this value and that for k_2 (10·5 sec.$^{-1}$) we can conclude that reoxidation of the enzyme is approximately twice as fast (k_4 = 21·5 sec.$^{-1}$). The ratio of the rates of reduction and reoxidation is in reasonable agreement with the steady-state ratio of reduced and oxidized enzymic FAD, as shown in Fig. 1.

The overall turnover of 8 (± 0·5) sec.$^{-1}$ at 25°, calculated from our experiments on the xanthine oxidase-catalysed formation of uric acid under optimum conditions, is compatible with Morell's (1952) value of 5·22 sec.$^{-1}$ at 19°. If the overall rate of hydrogen transfer from xanthine to enzymic FAD and from enzymic FAD to oxygen is determined by the two steps characterized by k_2 and k_4, then the rate constant k_0 for the overall reaction is given by $1/k_0 = 1/k_2 + 1/k_4$. Substituting the values given above for k_2 and k_4 we obtain k_0 = 7·88 sec.$^{-1}$. This is in agreement with the hypothesis that the overall reaction of the enzyme is determined by the two successive steps of reduction and reoxidation observed by the techniques described.

We can conclude, from the good agreement between the values calculated from the constants assigned to individual steps and those found experimentally for the steady-state position and the turnover rate of the enzyme, that the scheme suggested for the reaction is correct and that the individual steps postulated are necessary and sufficient to describe the process. The determinations of the Michaelis constants for both xanthine and oxygen are not intended to be more than approximations, but the finding that both these substrates form an enzyme–substrate compound before their respective oxidation and reduction is substantiated by the fact that these oxidation and reduction steps are first-order and independent of xanthine and oxygen concentrations over a wide range. The rather primitive method used to determine K_m in the fast-reaction apparatus could give erroneous results due to inhibition by reaction products. We have, however, ascertained that these errors cannot be serious. We have also found that substrate inhibition investigated by Hofstee (1955) did not affect our experiments. Hydrogen peroxide has an inhibitory effect on the enzyme, but only if the enzyme and hydrogen peroxide are in contact for some minutes. This phenomenon prevented us from interpreting the results of measurements of the rate of reoxidation of xanthine-reduced enzyme.

Results of various experiments with isotopes have given interesting information about the mechanism of xanthine oxidase reactions. Mason (1957) has reported that molecular oxygen is used in the formation of hydrogen peroxide during the reoxidation of the enzymic $FADH_2$ and that xanthine oxidase behaves in this respect like uricase (Bentley & Neuberger, 1952) and notatin (Bentley & Neuberger, 1949). The investigations into the stereospecificity of hydrogen transfer by xanthine oxidase did not produce such decisive results as those carried out with DPN-linked enzymes. This is due to the rapid exchange of the hydrogen atoms of reduced $FADH_2$ with the medium. Vennesland (1956) did, however, get some evidence that during the oxidation of acetaldehyde by xanthine oxidase hydrogen is transferred directly from aldehyde to the electron acceptor. Our measurements of the anaerobic reduction (k_2) and the overall rate (k_0) in 50 % (v/v) ^2H_2O showed

that the rate of reduction was not changed by deuterium in the medium, while k_0 was smaller by a factor 0·643. We have drawn the conclusion that the decrease of k_0 in the presence of D_2O is due to a decrease of k_4, the rate constant for the reoxidation. The most plausible explanation for this effect is the rapid exchange of $FADH_2$ with the medium and a slower rate of reoxidation for FAD^2H_2. This point, as well as the rate of exchange of hydrogen of reduced $FADH_2$ with the medium, is being investigated further. A more detailed analysis of the behaviour of flavoproteins may throw some light on the effect of the presence of deuterium on the respiratory chain. Experiments with free FAD should also prove useful. The differences between the reactivity and physical properties of free FAD and enzyme-bound FAD should be investigated; deuterium exchange may give further information on this point. In this connexion our measurements of the reduction of xanthine oxidase by 0·025 M-sodium dithionite are of interest. The reduction of the enzyme was rather slow, giving a pseudo-first-order reaction ($k = 0{\cdot}12$ sec.$^{-1}$). Some preliminary experiments (Gutfreund, unpublished observations) have shown that the reduction of free FAD by sodium dithionite under identical conditions is considerably faster ($k = 100$ sec.$^{-1}$).

Under the conditions of our experiments no semiquinone intermediates were detected. From the intensities of the absorption bonds reported for such intermediates by Beinert (1957) one would not expect to find them under the conditions used by us. It will require a good deal of further experimental work to determine the molar extinction coefficients of the semiquinone bands in flavoprotein systems, and a subsequent calculation of the concentrations and rate of formation and decomposition of such intermediates, before one can draw any conclusions about their role on the direct path of flavoprotein-catalysed hydrogen transfer. The presence of a free-radical intermediate during some reactions of xanthine oxidase has been observed by electron-spin resonance as described by Bray, Malmström & Vänngard (1959). At this stage we should like to abstain from any discussion of the detailed reaction mechanism. Further experiments to be carried out in collaboration with Dr Bray and his colleagues will show whether a hydride ion transfers two electrons from xanthine to enzymic FAD or whether the two electrons are transferred in distinguishable steps.

SUMMARY

1. The individual steps in the hydrogen transfer from xanthine to xanthine oxidase and from xanthine oxidase to oxygen were studied by fast-reaction spectrophotometric techniques. The reduction and reoxidation of the enzymic flavin-adenine dinucleotide was followed by observation of extinction value changes at 450 mμ and the appearance of uric acid at 310 mμ.

2. From studies over a wide range of concentrations of xanthine and oxygen we found that a very rapid combination of xanthine with enzyme is followed by the reduction of enzymic flavinadenine dinucleotide which is characterized by a first-order constant ($k_2 = 10{\cdot}5 \pm 0{\cdot}5$ sec.$^{-1}$). The reoxidation of the flavinadenine dinucleotide, which is preceded by a rapid combination with oxygen, is characterized by $k_4 = 21{\cdot}5 \pm 2$ sec.$^{-1}$.

3. Approximate values for the Michaelis constants for xanthine and oxygen, as well as the turnover number of the enzyme, have been determined from measurements of the rate of formation of uric acid, and a cyclic reaction scheme for xanthine oxidase is presented.

4. The effect of deuterium on the reactions of xanthine oxidase has been studied and has been provisionally interpreted.

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REFERENCES

Avis, P. G., Bergel, F. & Bray, R. C. (1955). *J. chem. Soc.* p. 1100.
Avis, P. G., Bergel, F., Bray, R. C., James, D. W. F. & Shooter, K. V. (1956). *J. chem. Soc.* p. 1212.
Beinert, H. (1957). *J. biol. Chem.* **225**, 465.
Bentley, R. & Neuberger, A. (1949). *Biochem. J.* **45**, 584.
Bentley, R. & Neuberger, A. (1952). *Biochem. J.* **52**, 694.
Bergel, F. & Bray, R. C. (1958). *Symp. biochem. Soc.* **15**, 64.
Bray, R. C., Malmström, B. G. & Vänngard, T. (1959). *Biochem. J.* (in the Press).
Chance, B. (1951). *Advanc. Enzymol.* **12**, 153.
Gibson, Q. H. (1952). *J. Physiol.* **117**, 49*P*.
Gutfreund, H. (1955). *Disc. Faraday Soc.* **20**, 167.
Hofstee, B. H. J. (1955). *J. biol. Chem.* **216**, 235.
Kalckar, H. M. (1947). *J. biol. Chem.* **167**, 429.
Mackler, B., Mahler, H. R. & Green, D. E. (1954). *J. biol. Chem.* **210**, 149.
Mason, H. S. (1957). *Science*, **125**, 1185.
Morell, D. B. (1952). *Biochem. J.* **51**, 657.
Nicholas, D. J. D. (1957). *Nature, Lond.*, **179**, 800.
Spencer, T. & Sturtevant, J. M. (1959). *J. Amer. chem. Soc.* **81**, (in the Press).
Theorell, H. & Chance, B. (1951). *Acta chem. scand.* **5**, 1127.
Vennesland, B. (1956). *J. cell. comp. Physiol.* **47**, 201.

The Activation of an Arylsulphatase from Ox Liver by Chloride and other Anions

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A few years ago an attempt was made by the senior author to purify the enzyme in ox liver which hydrolyses *p*-nitrophenyl sulphate. Considerable difficulty was experienced at first in obtaining consistent and intelligible activity figures during fractionation. The anomalies were found to be due to two factors: the enzyme was strongly inhibited by inorganic phosphate, so that the activity was appreciably reduced even by the amount of phosphate present in an aqueous extract of acetone-dried ox-liver powder; and the enzyme showed very little activity in the absence of chloride, so that it appeared to be lost on dialysis against water. Reasonable yields were obtained if activity determinations were carried out on samples dialysed against sodium chloride solution.

Since the work commenced, other purifications of arylsulphatase from ox liver have been reported, especially that of Roy (1953*b*) (arylsulphatase A) and Roy (1954) (arylsulphatase B). The latter enzyme has also been purified from human liver by Dodgson & Wynn (1958). However, the activation by chloride seemed of some interest and a detailed study of the effect of anions was undertaken; the results are reported in this paper.

In a number of cases it has been reported that the activity of arylsulphatases is affected by chloride. Dodgson, Spencer & Thomas (1953) stated that the particulate enzyme obtained in the course of fractionation studies with rat-liver homogenates was activated by potassium chloride, but later Roy (1953*b*) attributed this to 'a change in the state of dispersion of the enzyme studied by these authors'. Roy (1955) published pH curves obtained in the presence and in the absence of 0·08 M-chloride for both arylsulphatase A and B from ox liver. With arylsulphatase A the main effect is an inhibition by chloride of up to 10% at pH values above the optimum, which is shifted towards the acid side. The optimum pH for arylsulphatase B is also shifted by chloride, from pH 6·0 to 5·7; there is little change of velocity at the optimum pH, but there is about 30% activation by chloride at pH 5·8. The substrate used in Roy's experiments was nitrocatechol sulphate. Contrary to these findings for ox liver, Dodgson & Wynn (1958) found that arylsulphatase B from human liver was considerably inhibited by chloride. Sodium chloride (0·01 M) produces 10% increase in activity of the arylsulphatase prepared from limpet viscera by Dodgson & Spencer (1952).

The stimulation of nitrophenylsulphatase activity by chloride and other ions reported in the present paper is a much larger effect than any hitherto reported for arylsulphatases.

MATERIALS AND METHODS

Preparation of enzyme

Fresh ox liver was acetone-dried; 5 lb. batches were usually used. The powder was finally dried over conc. H_2SO_4 and NaOH; the activity of the powder was stable over a period of months. A portion (600 g.) of powder was extracted with 10 l. of 0·1 M-acetate buffer, pH 5·1, with mechanical stirring for 2 hr.; after centrifuging the residue was stirred up with a further 2·5 l. of water and spun again. The combined dark-red extracts were brought to pH 6·0 and solid $(NH_4)_2SO_4$ was added to 45% saturation. After standing overnight at 4° the precipitate was centrifuged off and the supernatant brought to 70% saturation with solid $(NH_4)_2SO_4$. After standing again for 12 hr. at 4° the precipitate was centrifuged down in a refrigerated centrifuge, and it was dissolved in the minimum amount of water and dialysed against two changes of distilled water, one of 0·1 M-NaCl and a further one of distilled water. The solution was adjusted to pH 4·0 with N-HCl, heated to 38° over a period of 10 min. and held at 38° for a further 30 min. During this period a small precipitate formed. The preparation was brought to pH 5·0 with N-NaOH and the heavy dark-coloured precipitate spun off at 0° and rejected; the supernatant was now adjusted to pH 6·0 with N-NaOH (all pH values were measured with the glass electrode). To this was added 0·5 vol. of calcium phosphate gel (dry wt. 30 mg./ml.), which was spun off at 0° after 5 min. and rejected. The supernatant was brought to 70% saturation with solid $(NH_4)_2SO_4$, and allowed to stand overnight at 4° and centrifuged. The precipitate was suspended in water and dialysed successively against water, 0·01 M-acetate at pH 6·0, water, 0·01 M-acetate at pH 6·0 and two changes of glass-distilled water. A small precipitate was spun off, giving 300–400 ml. of clear straw-coloured enzyme. This stock enzyme, which was normally diluted five times for use in the kinetic studies described in this paper, was kept frozen at −20° and was completely stable under these conditions for at least 6 months.

Substrates, activators and inhibitors

Monopotassium *p*-nitrophenyl sulphate (NPS) was supplied by Sigma Chemical Co., St Louis, Mo., U.S.A. Estimation by acid hydrolysis of three different samples used in this work gave values (after correcting for free *p*-nitrophenol) of 84, 98 and 103% of the theoretical for $NO_2 \cdot C_6H_4 \cdot OSO_3K$. The free nitrophenol in these batches corresponded to 0·22, 2·7 and 0·1% hydrolysis respectively. Stock solutions of NPS for the enzymic experiments were adjusted to pH 5·6 with acetic acid or NaOH.

Dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, NCS) was prepared by Mr B. Slater by the method of Roy (1953*a*). It contained 0·5% of free nitrocatechol and gave 94% of the theoretical amount of nitrocatechol on acid hydrolysis, assuming the preparation to be the dihydrate. Such preparations have been shown to contain appreciable amounts of nitropyrogallyl disulphate (Dodgson & Spencer, 1956; Roy & Kerr, 1956).

NaCl, KCl, KI, KNO_3, KSCN, KCN, Na_2SO_4 and citric acid, as well as the components of phosphate and acetate buffers, were AnalaR reagents. NaBr, NaF and $KClO_3$ were normal laboratory reagents supplied by British Drug Houses Ltd. or Hopkin and Williams Ltd.

Measurement of enzymic activity

Hydrolysis of NPS. In following the purification of the enzyme, and during some preliminary experiments, activity was measured by a method based on that of Huggins & Smith (1947). A portion (1 ml.) of M-acetate buffer (pH 5·1 unless otherwise stated), 1 ml. of M-NaCl and a suitable amount of enzyme were made up to 4 ml. and equilibrated at 38°. The reaction was started with 1 ml. of 6 mM-NPS and, after 1 hr., 5 ml. of N-NaOH was added; the *p*-nitrophenol was estimated in the Beckman Spectrophotometer at 400 mμ.

A method based on the amount of product formed in a fixed time is unsatisfactory for kinetic studies, especially if rather long times of incubation are necessary. For all of the activation and inhibition studies described in this paper, the Beckman Ratio Recording Spectrophotometer DK-2 (Beckman Instruments Inc., Fullerton, Calif., U.S.A.) was used.

Fig. 1 shows the absorption spectrum of NPS and the corresponding free phenol in acetate buffer, pH 5·6. The peak in the near-ultraviolet is shifted from 278 mμ in the sulphate to 318 mμ in the free phenol. The biggest increase in absorption at pH 5·6 on hydrolysis of NPS is shown at about 330 mμ; it was decided to use the change in extinction at 330 mμ as a measure of arylsulphatase activity. Because of the large absorption by substrate at this wavelength, all measurements were made against corresponding control solutions of NPS and the 20-fold photomultiplier was employed.

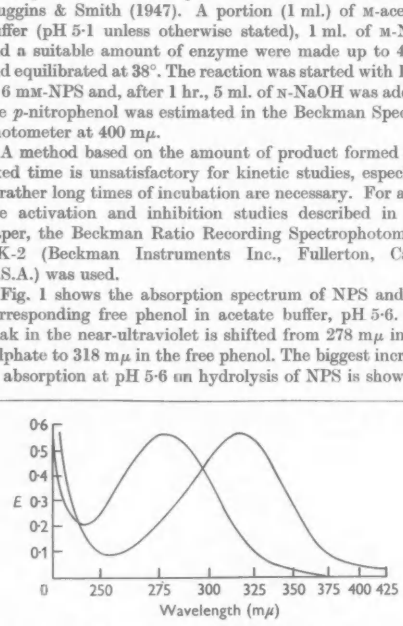

Fig. 1. Absorption spectrum of NPS and free *p*-nitrophenol. Spectra of 0·06 mM solutions in 0·2M-acetate, pH 5·6, measured at 38° in 1 cm. cells in the Beckman Ratio Recording Spectrophotometer.

Reactions were carried out in a total volume of 3 ml. in 1 cm. quartz cells in the Beckman 92527 Temperature Regulated Cell Holder at 38°. The standard reaction mixture contained 0·5 ml. of M-acetate buffer, 0·5 ml. of M-NaCl, enzyme and 1 ml. of 6 mM-NPS (added last to start the reaction). The control cell contained a similar mixture except that the enzyme was replaced by water. Typical progress curves are shown in Fig. 2. There was always a rapid apparent increase in extinction for 60–90 sec.; this does not correspond to stage I of the anomalous arylsulphatase A kinetics studied by Baum, Dodgson & Spencer (1958), but is an artifact, since a similar progressive rise for a period of 2–3 min. is shown when *p*-nitrophenol is pipetted into NPS in acetate buffer. After this initial period the increase in extinction was linear for at least 15 min.; the slope of the curve between 2 and 8 min. was used as a measure of the rate of enzymic hydrolysis. Separate calibration constants were needed at each pH value and at each substrate concentration, since the absorption by nitrophenol at 330 mμ is somewhat reduced in the presence of high concentrations of NPS. Calibration curves were prepared from mixtures of standard *p*-nitrophenol and NPS in acetate buffer; in each case Beer's Law was obeyed over the range of concentrations used. At pH 5·6 in 2 mM-NPS a change of extinction of 1 at 330 mμ corresponded to the production, in 3 ml. incubation mixtures, of 67·5 μg. of *p*-nitrophenol. Enzymic hydrolysis

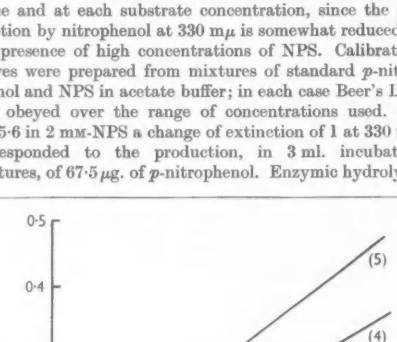

Fig. 2. Progress curves for enzymic hydrolysis of NPS. A tracing of the record of an individual experiment with the Beckman Ratio Recording Spectrophotometer. Reaction mixtures (3 ml.) contained 0·17M-acetate buffer, pH 5·1, 0·17M-NaCl, mM-NPS and the following amounts of stock enzyme diluted five times: (1) 0·2 ml.; (2) 0·3 ml.; (3) 0·5 ml.; (4) 1·0 ml.; (5) 1·5 ml. The time base of the machine was driven by a 60-cycle synchronous motor by 50-cycle current and the divisions on the time scale represent intervals of 144 sec., except for curve 1, for which they represent 72 sec.

rates were proportional to enzyme concentration at least up to amounts of enzyme giving rates of 160 μg. of *p*-nitrophenol/hr. With this standard test the activity of various batches of the stock solution of enzyme described above ranged from 600 to 800 μg. of nitrophenol/hr./ml. of enzyme.

Hydrolysis of NCS. It has not proved possible to devise a continuous-recording procedure for following NCS hydrolysis, and a method based on that of Roy (1953*a*) was used. The enzymic reactions were carried out in a total volume of 3 ml. containing 0·5 ml. of M-acetate, pH 5·6, enzyme and 0·5 ml. of 0·05 M-NCS (added last to start the reaction), at 38°. Incubation periods of either 10 or 15 min. were normally used, and the reaction was stopped by adding 5 ml. of fresh alkaline quinol reagent, prepared as described by Roy. Deproteinization was unnecessary with the purified enzyme and the phosphotungstic acid was omitted. The liberated nitrocatechol was determined spectrophotometrically at 520 mμ, the extinction being measured against corresponding controls in which the enzyme was added after the alkaline quinol. Fig. 3 shows that hydrolysis rates as measured by this procedure were proportional to enzyme concentration at least up to 0·2 ml. of the stock enzyme. The specific activities of various batches towards NCS at pH 5·6 in the absence of chloride ranged from 5000 to 6600 μg. of nitrocatechol/hr./ml. of stock enzyme.

Sulphate liberation. For most of the work hydrolysis was followed by the liberation of free phenol, but for the 'mixed-substrate' experiment sulphate production was followed by the method of Dodgson & Spencer (1953). The coloured solution obtained according to this procedure after the final centrifuging was diluted fourfold and the extinction at 500 mμ measured in the Beckman Spectrophotometer. A calibration curve was constructed by using measured amounts of standard K_2SO_4 solution containing up to 80 μg. of SO_4^{2-}.

RESULTS

Activation by anions

Activation by chloride of the enzymic hydrolysis of NPS. Fig. 4 shows the dependence of the activity of stock enzyme preparation on added NaCl at pH 5·1. All the early experiments were carried out at this pH, which is close to the optimum of 4·9 in acetate buffer reported by Roy (1953*b*) for arylsulphatase A. At this pH maximum activity is shown at about 0·2 M-NaCl, the activity falling off slightly at higher concentrations. Half the maximum velocity is obtained at about 0·02 M-NaCl. For the standard enzyme assay 0·17 M-NaCl was chosen.

Specificity of activating anions. A number of univalent anions were found to be effective as activators. The activation curves for these ions at pH 5·6 are shown in Fig. 5. Of other univalent

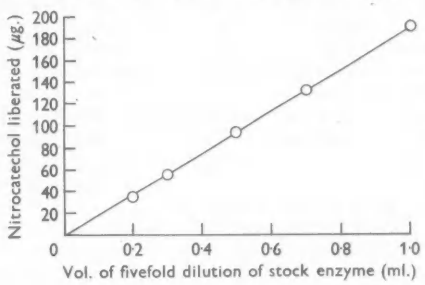

Fig. 3. Effect of enzyme concentration on NCS hydrolysis. Procedure was as described in the text; reaction mixtures contained 0·17 M-acetate, pH 5·6, 0·17 M-NaCl and 8·3 mM-NCS, with the amounts of enzyme shown. Incubation was at 38° for 10 min.

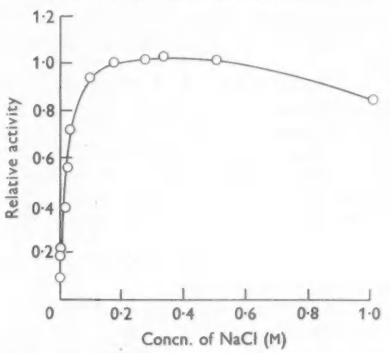

Fig. 4. Effect of chloride concentration on enzymic hydrolysis of NPS at pH 5·1. Reaction mixtures (3 ml.) contained 0·17 M-acetate buffer, mM-NPS and 0·2 ml. of stock enzyme. Rates of hydrolysis were measured in the Recording Spectrophotometer at 38° and are expressed relative to that in 0·17 M-NaCl.

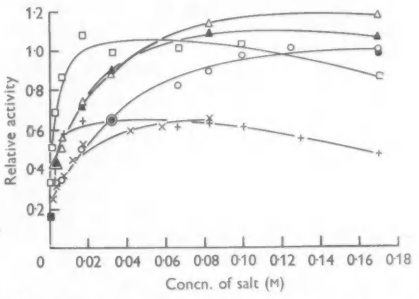

Fig. 5. Effect of a number of salts on enzymic hydrolysis of NPS at pH 5·6. Reaction mixtures (3 ml.) contained 0·17 M-acetate buffer, 2 mM-NPS and 0·2 ml. of stock enzyme. Rates of hydrolysis at 38° are expressed relative to that in a control containing 0·17 M-NaCl. ■, No added salt (mean of nine experiments); ○, NaCl; ●, KCl; △, NaBr; ▲, KI; □, KSCN; ×, $KClO_3$; +, KNO_3.

ions investigated, cyanide has no effect and fluoride is a powerful inhibitor (see below). Most of the activation experiments were carried out in 0·17 M-acetate buffer and the question arises whether acetate ion has any influence on the activity. In an experiment with enzyme and substrate carefully adjusted to pH 5·6, the same rates were obtained in the absence of buffer and in 33 mM-, 67 mM-, 0·17 M- and 0·33 M-acetate; thus there was no evidence that acetate ion was capable of activating the enzyme. No evidence was seen that acetate had any effect on the activation by chloride.

The enzyme was unaffected by Ca^{2+} or Mg^{2+} ions, and the nature of the alkali-metal ion present appeared to have no effect. Fig. 5 shows that the same activity was obtained in the presence of KCl or NaCl.

Interpretation of Fig. 5 in terms of affinity constants is complicated by the inhibition shown by higher concentrations of some of these salts, especially thiocyanate, nitrate and, to a lesser extent, iodide. At concentrations lower than those at which this inhibition becomes noticeable, the curves are more or less rectangular hyperbolas. It was found that by plotting the reciprocal of the activity (corrected for the small activity without added salt) against the reciprocal of the concentration of salt added, straight-line portions could be obtained; the reciprocals of the intercepts obtained by extrapolating these straight-line portions are shown in Table 1. The extrapolated maximum velocities are probably not significantly different, with the exception of that for chlorate; unfortunately this ion was not tested at higher concentrations because of the limited solubility of $KClO_3$. With nitrate, although the data at low concentrations extrapolate to the same maximum velocity as with chloride, the maximum velocity actually obtained is only 0·6 of the maximum obtained in chloride, because of the inhibition progressively shown by nitrate in concentrations above 0·05 M. On the other hand the apparent affinities are markedly different, the affinities increasing in the order: $Cl^- < Br^- < NO_3^- < ClO_3^- < I^- < SCN^-$. The more efficient activating ions are the ones which most markedly show inhibition at high concentrations. From a purely practical point of view the most efficient activators would appear to be thiocyanate or bromide.

Activity in the absence of activating anions. The stock preparation of enzyme invariably showed some activity in the absence of added NaCl, even after prolonged dialysis. The figure used as the origin of the curves in Fig. 5, i.e. an activity in the absence of salt of 0·16 of that in 0·17 M-NaCl, represents the mean of nine separate experiments. Although this is quite low, it is of some interest to know whether this represents a real activity of the ion-free enzyme or an activation by anions already present in the preparation. On the assumption that this residual activity might be due to traces of firmly bound chloride in the enzyme, an attempt was made to remove it by treatment with anionic-exchange resins, both in batches and on columns. In parallel experiments De-Acidite F, Amberlite IRA-400 and Dowex 2, in acetate form, were used There was a small increase in specific activity (which might be due to removal of inhibitors), but there was no significant change in the ratio of activity without added salt to activity in 0·17 M-NaCl.

There remains the possibility that this low activity is produced by salts present in the reagents used for assay. It has been mentioned above that it is unlikely that the acetate used as a buffer has any activating power; the same activities were obtained in the absence of added buffer, when the only acetate present arose from the traces of acetic acid used in adjusting the pH of enzyme and substrate. It is possible, however, that the NPS used contained traces of chloride or other activator. Some support for this possibility is given by the fact that different values for the intrinsic activity were obtained with different batches of commercial NPS. For three batches of substrate the activities obtained in the absence of salt were 9·0, 18·5 and 11·0 % respectively of those in 0·17 M-NaCl. Presumably the lowest of these figures represents the maximum possible value for the intrinsic activity of anion-free enzyme.

Effect of univalent anions on enzymic hydrolysis of NCS. At an early stage in the work it was found that at no stage in the purification was there any activation of NCS hydrolysis by chloride parallel to that of NPS hydrolysis. In fact at pH 5·6 NaCl is an inhibitor of NCS hydrolysis, as shown in Fig. 6. The implications of this observation are discussed below.

Table 1. *Apparent activator constants and relative maximum velocities of activating anions*

Values obtained from intercepts of reciprocal plots of the data shown in Fig. 5; the apparent activator constant represents the concentration of activator at which half the extrapolated maximum velocity ($V_{max.}$) is attained.

Anion	Apparent activator constant (mM)	$V_{max.}$ (relative to that in Cl^-)
Cl^-	21	1·0
Br^-	16	1·16
I^-	6	1·0
ClO_3^-	8	0·42
NO_3^-	10	1·0
SCN^-	4	1·16

Nature of the activation

In an attempt to obtain information which might throw light on the mechanism involved, the interaction of activator concentration and other variables on the enzyme activity was investigated.

pH *and activation.* Fig. 7 shows the activity–pH curve in 2 mM-NPS in the presence and in the absence of 0·17 M-NaCl; Fig. 8 shows the corresponding curves in 17 mM-NCS. It will be seen at once that the chloride activation of NPS hydrolysis cannot be explained in terms of a shift in the dissociation constants controlling the pH curve. There may be a small shift in the optimum pH from about 5·9 in the absence of chloride to 5·6 in 0·17 M-NaCl, although it is not certain that this is significant in view of the difficulty in measuring the low activities obtained in the absence of chloride. But even if the activities are compared at their

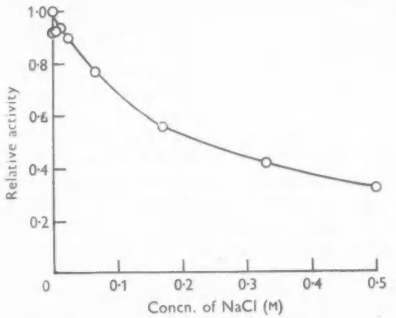

Fig. 6. Effect of chloride concentration on enzymic hydrolysis of NCS at pH 5·6. Reaction mixtures (3 ml.) contained 0·17 M-acetate buffer, 83 mM-NCS and 0·06 ml. of stock enzyme. Hydrolysis rates, measured by nitrocatechol production in 10 min., are expressed relative to that without chloride.

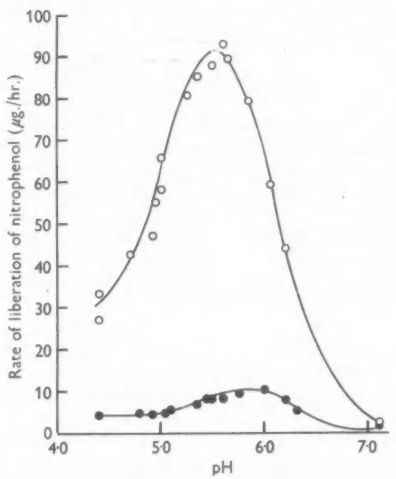

Fig. 7. Effect of pH on enzymic hydrolysis of NPS, in the presence and in the absence of chloride. Reaction mixtures (3 ml.) contained 0·17 M-acetate buffer, 2 mM-NPS and 0·2 ml. of stock enzyme. ●, No chloride; ○, in 0·17 M-NaCl.

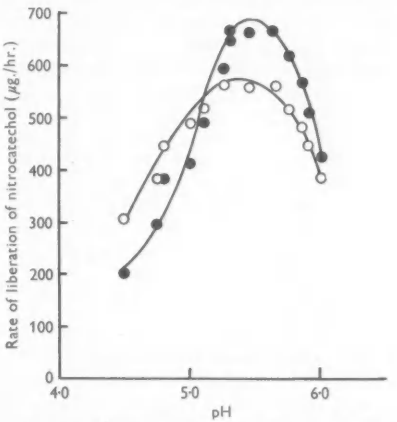

Fig. 8. Effect of pH on enzymic hydrolysis of NCS, in the presence and in the absence of chloride. Reaction mixtures (3 ml.) contained 0·17 M-acetate buffer, 17 mM-NCS and 0·1 ml. of stock enzyme. ●, No chloride; ○, in 0·17 M-NaCl.

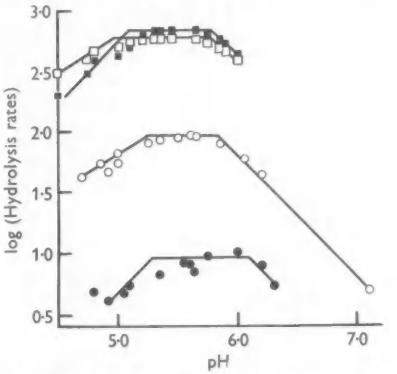

Fig. 9. Dixon plots of data in Figs. 7 and 8. Logarithms of rates of phenol liberation by 0·1 ml. of stock enzyme are plotted against pH. ●, NPS, no chloride; ○, NPS, 0·17 M-NaCl; ■, NCS, no chloride; □, NCS, 0·17 M-NaCl.

appropriate optimum pH, the activation by chloride is still very marked, being increased about 9·2 times by 0·17 M-NaCl. In NCS hydrolysis, there is a small shift of the optimum in the same direction, from about 5·5 without chloride to 5·4 in NaCl. Both sides of the curve are shifted, so that although chloride inhibits at the optimum pH, it shows slight activating properties below pH 5·1.

Fig. 9 shows Dixon plots (Dixon, 1953; Dixon & Webb, 1958, p. 136) of the pH data. Their interpretation must be made with some reserve, since sub-optimum concentrations of substrate were used and hence true V values were not obtained; but they suggest functional groups in the enzyme with pK values of about 5·2 and 5·9 in chloride, and perhaps rather higher in the absence of chloride.

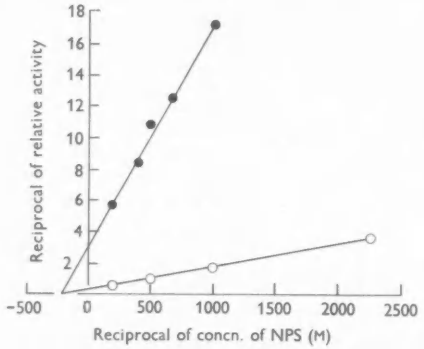

Fig. 10. Effect of chloride on the affinity of arylsulphatase for NPS at pH 5·6. Lineweaver–Burke plots; reaction mixtures (3 ml.) contained 0·17 M-acetate, pH 5·6, and 0·2 ml. of stock enzyme. ●, No chloride; ○, in 0·17 M-NaCl.

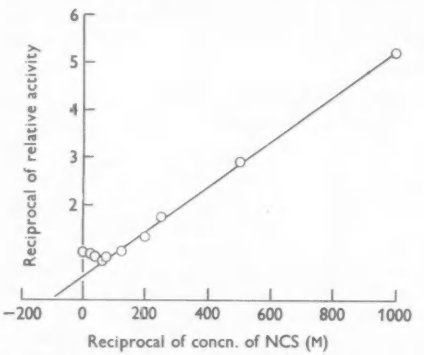

Fig. 11. Lineweaver–Burke plot for the hydrolysis of NCS at pH 5·6. Reaction mixtures (3 ml.) contained 0·17 M-acetate buffer and 0·1 ml. of stock enzyme.

Interaction of activator and substrate. The effect of substrate concentration on the activity towards NPS was determined in the presence and in the absence of 0·17 M-NaCl; the corresponding Lineweaver–Burke plots are shown in Fig. 10. This showed that chloride has no effect on the affinity for NPS, K_m being about 4·4 mM in both. The affinity for NPS is appreciably greater than that for NCS in the absence of chloride. Fig. 11 shows K_m for NCS at pH 5·6 about 0·01 M; a similar K_m is obtained in the presence of 0·17 M-NaCl.

Thus chloride appears to act by a true increase in V, i.e. in the rate of breakdown of the activated substrate at the optimum pH.

Effect of anions on the specificity of the enzyme

In characterizing arylsulphatases, use has been made by a number of authors of the specificity pattern, and in particular of the relative activity towards NPS and NCS (see Discussion). It will be clear from the results given above that the ratios obtained with the enzyme studied vary with the salt present. Thus if the activities are compared at their respective optimum pH values, but at the arbitrary substrate concentrations used in the standard tests (17 mM-NCS and 2 mM-NPS), the ratio of activities (NCS/NPS) falls from 135 in the absence of chloride to 12 in 0·17 M-NaCl. If the extrapolated maximum velocities from the Michaelis curves are compared, the ratio of activity NCS/NPS at pH 5·6 is 55 in the absence of salt and 3·3 in 0·17 M-NaCl.

It is, of course, desirable to establish whether these two arylsulphatase activities, differing so greatly in their response to salt, are due to a single enzyme. The ratio of activity to the two substrates does not change appreciably during the last three stages of the purification, and is not altered by further attempted fractionation by precipitation with $(NH_4)_2SO_4$ or acetone, or by adsorption on calcium phosphate gel. Further evidence of identity was obtained in two ways.

Effect of inhibitors. Fluoride and a number of multivalent anions inhibit both activities. Fig. 12 shows inhibition curves on a logarithmic scale for a number of salts for (*a*) NPS hydrolysis and (*b*) NCS hydrolysis. The inhibition by fluoride of both activities runs more or less parallel with a pI_{50} (negative logarithm of the concentration giving 50% inhibition) of 3·4. Phosphate and sulphate show the same relative activity in the two cases, but are both more effective against the hydrolysis of NPS than of NCS under the conditions studied. The pI_{50} values for phosphate and sulphate are 4·1 and 2·3 respectively against NPS hydrolysis and 3·4 and 1·6 against NCS hydrolysis. These results

would be compatible with a single enzyme hydrolysing both substrates, if phosphate and sulphate were competitive and fluoride was a non-competitive inhibitor. An attempt was made to verify this in the usual way. The results, although consistent with this possibility, were not entirely conclusive, as non-linear Lineweaver–Burke plots were obtained in the presence of inhibitors.

An alternative possible explanation of the action of the inhibitors mentioned is that they compete with the activating ion. This was ruled out by experiments in which the inhibition of NPS hydrolysis by 0·083 mM-phosphate, 6·7 mM-sulphate and 0·33 mM-fluoride was measured in the absence of NaCl and with added NaCl to a final concentration of 17 mM, 33 mM and 170 mM, in each case relative to corresponding controls without the inhibitor. For each of the inhibitors the percentage inhibition remained constant within experimental error (3–4 %); thus the apparent affinity for chloride is not affected by these inhibitors.

Hydrolysis of mixed substrates. The rate of enzyme action on a mixture of two substrates, compared with that on the two substrates present singly, has been much used, often in a semi-qualitative way, to determine whether two reactions are due to the same enzyme. An experiment of this kind was carried out with NPS and NCS; in this instance hydrolysis was followed by the liberation of sulphate, since this is the product common to the two reactions under investigation. The results of duplicate experiments are shown in Table 2. Qualitatively the experiment suggests a single enzyme, since the mixed-substrate velocity is only about 60 % of the sum of the velocities with the substrates attacked separately.

'Mixed-substrate' experiments have most commonly been carried out with the two substrates in equimolar concentrations. In the present experiments the substrates were used at different concentrations (8·3 mM-NCS and 2·0 mM-NPS) to ensure reasonably comparable velocities; the concentrations were in fact roughly related to the Michaelis constants. The result to be expected in such a case if only one enzyme is involved is best expressed in terms of 'relative concentrations'; it can be shown (Dixon & Webb, 1958, p. 92) that

$$v_t = \frac{V_a \alpha + V_b \beta}{1+\alpha+\beta},$$

where V_a and V_b represent the maximum velocities with excess of two substrates A and B, and v_t is the 'mixed-substrate velocity' when the relative concentrations (that is, the ratio of their concentrations to the respective Michaelis constants) of A and B are α and β. If v_a and v_b are the actual velocities when the two substrates at relative concentrations α and β are attacked singly, it can be shown, by substitution of the usual Michaelis expressions for these velocities into the equation above, that

$$v_t = \frac{v_a(1+\alpha)+v_b(1+\beta)}{1+\alpha+\beta}.$$

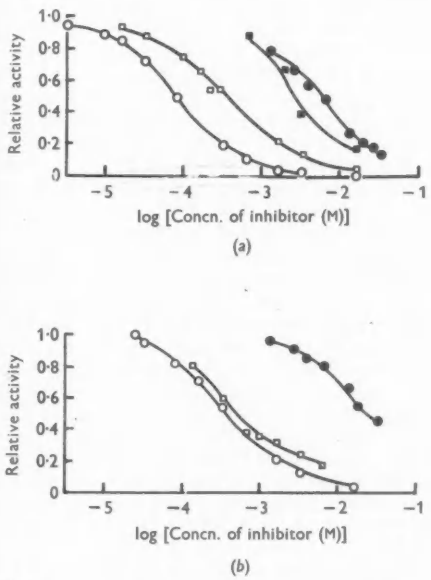

Fig. 12. Inhibition of arylsulphatase. (*a*) NPS hydrolysis; (*b*) NCS hydrolysis. Reaction mixtures (3 ml.) contained 0·17 M-acetate buffer, pH 5·6, and, for (*a*), 0·17 M-NaCl, 6 mM-NPS and 0·2 ml. of stock enzyme; for (*b*), 83 mM-NCS and 0·1 ml. of stock enzyme. ○, Phosphate; ●, sulphate; □, fluoride; ■, citrate.

Table 2. *Hydrolysis of* p*-nitrophenyl sulphate and 2-hydroxy-5-nitrophenyl sulphate by the enzyme, singly and in a mixture*

Hydrolysis by 0·2 ml. of stock enzyme in 0·17 M-acetate, pH 5·6, and 0·17 M-NaCl, at 38°, was measured by sulphate liberated in 30 min. In each case the result is corrected for free sulphate in the substrates and any non-enzymic hydrolysis by corresponding controls without enzyme.

Concn. of substrate	Sulphate produced in 30 min. (μg.) Expt. 1	Expt. 2	Mean
8·3 mM-NCS	121·5	126	124
2·0 mM-NPS	63	70·5	67
8·3 mM-NCS plus 2·0 mM-NPS	105	120	112·5

In the special case where the two substrates are mixed at the same relative concentration, i.e. $\alpha = \beta = \sigma$,

$$\frac{v_t}{v_a+v_b} = \frac{1+\sigma}{1+2\sigma}.$$

Thus the mixed-substrate velocity would be the sum of the velocities with separate substrates at very low relative concentrations, but would fall to two-thirds of the sum when σ is 1 and to half the sum when σ is very high. In the present experiment the mean relative concentration of the two substrates is 0·65, which should give a value of $v_t/(v_a+v_b)$ of 0·71. The experimental value of 0·6 is in reasonable agreement with this, considering the difficulty of the benzidine sulphate-assay method for sulphatase activity. It seems highly probable that a single enzyme in the preparation studied hydrolyses both NPS and NCS.

DISCUSSION

Although many enzymes are activated by cations, activation by anions is comparatively rare. The classical case is perhaps the activation of animal α-amylase by chloride discovered by Cole (1904) and investigated in detail by Myrbäck (1926). A number of univalent anions were effective but chloride was most efficient, the order of effectiveness being $ClO_3^- < NO_3^- < I^- < Br < Cl^-$. This is almost the reverse of the order of activity found with arylsulphatase. Another well-known case is that of fumarase, studied by Mann & Woolf (1930) and Massey (1953). For this enzyme multivalent ions were effective, arsenate being most active and phosphate, citrate, borate, arsenite, selenate and sulphate progressively less so; chloride was inhibitory. For both fumarase and α-amylase the effects of anions are readily seen, from the pH–activity curves, to be due to an effect on the dissociation of the ionizing groups at the active centre of the enzyme, which determine the optimum pH. The more effective activators for both enzymes raise the pK of the basic group involved but have little effect on the acidic group; with some other ions both pK values are raised so that the whole curve is displaced, producing an inhibition on the acid side and an activation on the alkaline side but little change in the maximum velocity. It seems probable from the pH curves presented in this paper that the activation of arylsulphatase is not of this type.

Another mechanism whereby ions might affect enzyme activity is by influencing the combination of enzyme and substrate. Theorell and his co-workers (Theorell, Nygaard & Bonnichsen, 1955; Theorell, 1958) found that, with liver alcohol dehydrogenase, chloride reduces the affinity for reduced coenzyme I by increasing the rate of dissociation from the enzyme, but decreases the rate of dissociation of the oxidized coenzyme–enzyme complex. Thus chloride can act as either an activator or an inhibitor of alcohol dehydrogenase according to the experimental conditions. Chloride has no effect on the affinity of arylsulphatase for either of the substrates studied, and it seems probable that we are dealing here with a direct effect of chloride on the breakdown of the enzyme–substrate complexes; no clear case of such an effect of anions has hitherto been established.

The relative rates of hydrolysis of various derivatives of phenyl hydrogen sulphate, and in particular NCS and NPS, have been much used recently as a criterion for distinguishing different arylsulphatases. Dodgson & Spencer (1957) proposed that arylsulphatases should be divided into two groups: type I enzymes, showing considerable affinity for and activity towards simple arylsulphatases such as NPS and *p*-acetylphenyl sulphate, but much less activity towards NCS, and being strongly inhibited by cyanide but unaffected by sulphate or phosphate; and type II enzymes, showing little activity towards NPS but considerably more towards disubstituted phenyl sulphates such as NCS, and being strongly inhibited by phosphate and sulphate but not cyanide. Of the three arylsulphatases from animal liver previously described, arylsulphatase C is a type I enzyme, and arylsulphatases A and B are type II enzymes. The last two enzymes were originally distinguished by Roy (1953*a*) by their action on NCS; arylsulphatase A shows an optimum pH of 4·9 and an optimum substrate concentration of 3 mM-NCS, whereas arylsulphatase B shows an optimum pH of 6·0 (or 5·7 in chloride) and an optimum substrate concentration greater than 0·03 M. Roy (1958), in comparative studies of the distribution of these enzymes in the livers of a number of species, used NPS and NCS as diagnostic substrates for arylsulphatase C and arylsulphatases A and B respectively. It is obvious that such criteria must be treated with great caution unless the ionic composition of the reaction medium is rigorously defined, in view of the possible profound effect of anions on the specificity pattern and optimum pH. Caution is particularly necessary when dealing with crude preparations which may contain appreciable amounts of chloride extracted from the tissue used as a source of enzyme.

The relationship of the enzyme described in this paper to arylsulphatases A, B and C of ox liver is not completely clear. The properties of these three enzymes have been tabulated by Roy (1956). The characteristics of the present enzyme agree most closely with those of arylsulphatase B, especially in optimum pH for NCS hydrolysis, inhibition by

phosphate, sulphate and fluoride and insensitivity to cyanide. The affinities for both NPS and NCS are, however, considerably greater than those reported for arylsulphatase B and are, in fact, close to those given for arylsulphatase C (which, however, is insoluble and sensitive to cyanide but not to sulphate). The NCS/NPS activity ratios are given by Roy as 20 for A, >50 for B and 0·5 for C [Dodgson & Wynn (1958) give 17 for human-liver arylsulphatase B]; all these values were obtained at low chloride concentration. The enzyme described here gives an NCS/NPS activity ratio of 55 in the absence of salt and 3·3 in chloride; this seems reasonably compatible with its identity with arylsulphatase B.

Other enzymes from animal tissues hydrolysing aromatic substances have been reported with properties not unlike those of arylsulphatase; for example Singer & Fruton (1957) showed that the same enzyme from spleen probably hydrolysed phenyl phosphates, phosphoramidate and phosphoprotein; this enzyme had an optimum pH near 6 and was inhibited by phosphate and fluoride. The relationship between the hydrolytic activities of animal tissues towards various synthetic substrates containing aryl groups is being investigated.

SUMMARY

1. The partial purification of an enzyme in ox liver hydrolysing *p*-nitrophenyl sulphate (NPS) and 2-hydroxy-5-nitrophenyl sulphate (NCS) is described.

2. The use of the Ratio Recording Spectrophotometer for following the progress of NPS hydrolysis is described.

3. The hydrolysis of NPS by the enzyme is strongly activated by chloride and some other univalent anions whereas the hydrolysis of NCS by the enzyme is inhibited by chloride.

4. The apparent affinities for activating anions, as observed by NPS hydrolysis, increase in the order Cl^-, Br^-, NO_3^-, ClO_3^-, I^-, SCN^-. The maximum velocity obtained with each ion except ClO_3^- is almost the same; but the velocity falls off at higher concentrations of thiocyanate, nitrate and iodide.

5. The activity of the enzyme towards NPS in the absence of added salts could not be reduced below 9% of that in 0·17 M-NaCl.

6. Chloride has little effect on the dependence of either NPS or NCS hydrolysis on pH.

7. Chloride has no effect on the affinity of the enzyme for either NPS or NCS.

8. Phosphate, sulphate and fluoride inhibit the action of the enzyme on both NPS and NCS; the effect is not due to competition with activator, but phosphate and sulphate may compete with substrate.

9. Mixed-substrate experiments suggest that both substrates are attacked by a single enzyme in the preparation.

10. The implications of this work for the characterization of sulphatases and the relationship of the present enzyme to the arylsulphatases previously described are discussed.

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REFERENCES

Baum, H., Dodgson, K. S. & Spencer, B. (1958). *Biochem. J.* **69**, 567.
Cole, S. W. (1904). *J. Physiol.* **30**, 202.
Dixon, M. (1953). *Biochem. J.* **55**, 161.
Dixon, M. & Webb, E. C. (1958). *Enzymes.* London: Longmans Green and Co.
Dodgson, K. S. & Spencer, B. (1952). *Biochem. J.* **51**, xliii.
Dodgson, K. S. & Spencer, B. (1953). *Biochem. J.* **55**, 436.
Dodgson, K. S. & Spencer, B. (1956). *Biochim. biophys. Acta*, **21**, 175.
Dodgson, K. S. & Spencer, B. (1957). *Meth. biochem. Anal.* **4**, 211.
Dodgson, K. S., Spencer, B. & Thomas, J. (1953). *Biochem. J.* **53**, xxxvi.
Dodgson, K. S. & Wynn, C. H. (1958). *Biochem. J.* **68**, 387.
Huggins, C. & Smith, D. R. (1947). *J. biol. Chem.* **170**, 391.
Mann, P. J. G. & Woolf, B. (1930). *Biochem. J.* **24**, 427.
Massey, V. (1953). *Biochem. J.* **53**, 67.
Myrbäck, K. (1926). *Hoppe-Seyl. Z.* **159**, 1.
Roy, A. B. (1953*a*). *Biochem. J.* **53**, 12.
Roy, A. B. (1953*b*). *Biochem. J.* **55**, 653.
Roy, A. B. (1954). *Biochem. J.* **57**, 465.
Roy, A. B. (1955). *Biochem. J.* **59**, 8.
Roy, A. B. (1956). *Biochem. J.* **64**, 651.
Roy, A. B. (1958). *Biochem. J.* **68**, 519.
Roy, A. B. & Kerr, L. M. H. (1956). *Nature, Lond.*, **178**, 376.
Singer, M. F. & Fruton, J. S. (1957). *J. biol. Chem.* **229**, 111.
Theorell, H. (1958). *Advanc. Enzymol.* **20**, 31.
Theorell, H., Nygaard, A. P. & Bonnichsen, R. (1955). *Acta chem. scand.* **9**, 1148.

The Mechanism of Action of Chlorpromazine

2. REDUCED DIPHOSPHOPYRIDINE NUCLEOTIDE–CYTOCHROME *c* REDUCTASE AND COUPLED PHOSPHORYLATION*

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In the previous paper (Dawkins, Judah & Rees, 1959) it was shown that 10-(3-dimethylaminopropyl)-2-chlorophenothiazine (chlorpromazine) was an inhibitor of cytochrome oxidase and also exhibited some action against oxidative phosphorylation. During the course of this investigation, it became apparent that chlorpromazine also had a second point of action in the respiratory chain. This paper describes experiments which indicate the site of action of chlorpromazine. Also included are some observations on the barbiturate 5-ethyl-5-*iso*amylbarbituric acid (Amytal), which has a very similar action (Ernster, 1956). A preliminary communication of these results has been published (Dawkins, Judah & Rees, 1958).

METHODS

Animals and enzyme preparations were identical with those described in the previous paper (Dawkins *et al.* 1959), as were all materials. Mitochondria were subjected to two types of treatment: (1) Pre-incubation in 0·075 M-sucrose for 30 min. at 0°. For this, mitochondria were suspended in 0·25 M-sucrose so that the mitochondria derived from 1 g. of liver were contained in 1 ml. of suspension. This was diluted with ice-cold water to give a final concentration of 0·075 M-sucrose. If required, the mitochondrial concentration was adjusted by dilution with cold 0·075 M-sucrose. (2) Pre-incubation in water. The mitochondrial pellets were suspended directly in cold water to make a 10% suspension and this was diluted with cold water as required. The suspension was held at 0° for at least 30 min. before use.

The reduction of cytochrome *c* by β-hydroxybutyrate and succinate was followed spectrophotometrically, with a Unicam SP. 500 spectrophotometer, by determining the increase in absorption at 550 mμ. The components of the system were as follows: substrate (sodium salt), 33 mM; cytochrome *c*, 40 μM; inorganic sodium orthophosphate buffer, pH 7·2, 33 mM; NaCN, 0·1 mM; ethylenediaminetetra-acetic acid (EDTA) neutralized to pH 7·2 with NaOH, mM. Mitochondria were added in a volume of 0·1 ml. Final volume was 3 ml. When reduced diphosphopyridine nucleotide (DPNH) was used as substrate, it was added at a concentration of 0·67 mM. Measurements were made at room temperature (20°) in cuvettes with a 1 cm. light path.

In manometric experiments with isolated mitochondria, the following medium was used: adenosine triphosphate (ATP), 1·7 mM; $MgSO_4$, 6·7 mM; KCl, 25 mM; glucose, 30 mM; yeast hexokinase, 0·05 ml.; inorganic sodium orthophosphate buffer, pH 7·2, 10 mM; cytochrome *c*, 20 μM; substrate, 10 mM. Mitochondria were added in 0·5 ml. of 0·25 M-sucrose, 0·075 M-sucrose or water as indicated. Final volume of flask contents was 3 ml. The centre well contained 0·1 ml. of 30% (w/v) KOH and filter paper. The temperature was 38° and the gas phase was air. Variations from these conditions are noted where relevant.

The exchange reaction between ^{32}P and ATP was studied in the following system: ATP, 2·5 mM; inorganic sodium orthophosphate buffer, pH 7·2, 5 mM; KCl, 25 mM; $MgSO_4$, 5 mM. ^{32}P equivalent to 15 000 counts/min. was added to each vessel; it was obtained from The Radiochemical Centre, Amersham, Bucks, and purified as described by Kennedy (1953). For the measurement of radioactivity a liquid counter (20th Century Electronics Ltd.; thin-walled B6) was used. The results were corrected for isotope decay, background and dead time of the instrument. Mitochondria equivalent to 1 mg. of N were added in 0·5 ml. of 0·25 M-sucrose. The final volume was 2 ml. and the tubes were incubated for 15 min. at 20°. 5-Ethyl-5-*iso*amylbarbituric acid (Amytal) was a commercial preparation. ^{32}P incorporated into the nucleotides was measured as described by Nielsen & Lehninger (1955). All the results quoted in this paper are representative of at least six similar experiments.

RESULTS

Inhibition of β-hydroxybutyrate oxidation

When chlorpromazine at a concentration of 0·2 mM is present with mitochondria isolated and suspended in 0·25 M-sucrose oxidizing β-hydroxybutyrate, inhibitions of oxygen uptake are seen (Table 1). At a concentration of 0·1 mM, however, little or no effect may be obtained. Pre-incubation of the particles in 0·075 M-sucrose rendered them more susceptible to chlorpromazine, so that 0·1 mM-inhibitor now produced 70% inhibition of oxygen uptake. This is interpreted as indicating a permeability barrier preventing the access of chlorpromazine to its site of action in the intact particles.

* Part 1: Dawkins, Judah & Rees (1959).

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Table 1. *Inhibition of β-hydroxybutyrate oxidation by chlorpromazine*

The system contained: ATP, 1·7 mM; $MgSO_4$, 6·7 mM; KCl, 25 mM; glucose, 30 mM; yeast hexokinase, 0·05 ml.; inorganic sodium orthophosphate buffer, pH 7·2, 10 mM; cytochrome *c*, 20 μM; β-hydroxybutyrate, 10 mM. Mitochondria (0·4 mg. of N) were added in 0·5 ml. of 0·25 M-sucrose or 0·5 ml. of 0·075 M-sucrose. The final volume of flask contents was 3 ml. The centre well contained 0·1 ml. of 30% (w/v) KOH and filter paper. Temperature was 38°. Gas phase was air. Readings were commenced after 7 min. thermal equilibration and were continued for 30 min. thereafter. Pre-incubation of mitochondria: mitochondria were held in 0·25 M-sucrose or in 0·075 M-sucrose for 30 min. at 0° before addition to the Warburg flasks.

Suspension medium for mitochondria	Concn. of chlorpromazine (mM)	Uptake of O_2 with chlorpromazine (μg.atom of O_2/mg. of N/hr.)	Uptake of O_2 of control (μg.atom of O_2/mg. of N/hr.)	Mean inhibition (%)
0·25 M-Sucrose	0·1	26·33 ± 1·25	26·83 ± 0·79	2·5
0·25 M-Sucrose	0·2	7·83 ± 1·79	25·75 ± 3·04	69·5
0·075 M-Sucrose	0·1	9·0 ± 1·19	29·66 ± 1·25	69·8

Table 2. *Action of chlorpromazine on phosphorylating and non-phosphorylating mitochondrial systems*

Components of the systems were: ATP, 1·7 mM; KCl, 25 mM; $MgSO_4$, 6·7 mM; inorganic sodium orthophosphate buffer, pH 7·2, 5 mM; ^{32}P (600 000 counts/min.); cytochrome *c*, 66 μM; NaCN, mM; ethylenediaminetetra-acetic acid, neutralized to pH 7·2 with NaOH, mM; 2-amino-2-hydroxymethylpropane-1:3-diol HCl buffer, pH 7·2, 33 mM; substrate (sodium salt), 33 mM. Mitochondria equivalent to 0·04 mg. of N were added in 0·1 ml. of water or 0·075 M-sucrose to start the reaction. Final volume was 3 ml.; temperature 20°. The appropriate blanks did not contain substrate and the results are corrected for blank values as described by Borgstrom, Sudduth & Lehninger (1955). The reaction was followed for 10 min. by observing the increase in extinction at 550 mμ in cells of 1 cm. light path. The esterification of inorganic phosphate was determined by the method of Nielsen & Lehninger (1955).

Pre-incubation medium for mitochondria	Substrate	Concn. of chlorpromazine (mM)	Cytochrome *c* reduced (μm-moles)	P esterified (μm-moles)	P/O	Percentage inhibition of electron transport
0·075 M-Sucrose	β-Hydroxybutyrate	Nil	12	7·8	1·3	—
0·075 M-Sucrose	β-Hydroxybutyrate	0·1	6	4·0	1·33	50
Water	β-Hydroxybutyrate + DPN (1 μmole)	Nil	50	Nil	—	—
Water	β-Hydroxybutyrate + DPN (1 μmole)	0·1	52	Nil	—	—
Water	β-Hydroxybutyrate	Nil	Nil	Nil	—	—
0·075 M-Sucrose	Succinate	Nil	53	7·5	0·3	—
0·075 M-Sucrose	Succinate	0·2	54	8·0	0·3	Nil
Water	Succinate	Nil	50	Nil	—	—
Water	Succinate	0·2	51	Nil	—	—

There appears to be a species variation in the mitochondrial permeability to chlorpromazine. Mitochondrial preparations from the livers of the Wistar strain of rats used in our Laboratory are apparently more permeable to chlorpromazine than preparations from Norwegian hooded rats.

In the previous investigation (Dawkins *et al.* 1959) we have shown that the inhibition of β-hydroxybutyrate oxidation is not relieved by a large excess of cytochrome *c*, and it seemed probable that the inhibition was not due to the effect of chlorpromazine on cytochrome oxidase. We therefore examined the effect of chlorpromazine on the reduction of cytochrome *c* by β-hydroxybutyrate. Table 2 shows the results of these experiments.

Mitochondria were pre-incubated in 0·075 M-sucrose for 30 min. at 0° to allow access of the added cytochrome *c* to the respiratory chain, since particles in 0·25 M-sucrose reduce cytochrome *c* very slowly under these conditions. It can be seen that chlorpromazine inhibits electron transport when β-hydroxybutyrate is the substrate, without affecting the efficiency of the coupled phosphorylation. When succinate is the substrate, there is no inhibition whatever. The P/O ratio for succinate is low, the theoretical figure for this span being 1·0. However, our results agree with those of Borgstrom, Sudduth & Lehninger (1955). It will be observed that the phosphorylative efficiency is given as a P/O ratio. This is calculated on the assumption that the reduction of cytochrome *c* is by a one-electron transfer. The ratios are therefore obtained by dividing the μm-moles of phosphate esterified by half the μm-moles of cytochrome *c* reduced.

When mitochondria were pre-incubated in water for 30 min. at 0° the phosphorylation was abolished (see also Lehninger, 1953–54; Judah & Williams-Ashman, 1951), the rate of reduction of cyto-

chrome *c* was accelerated and an absolute requirement for diphosphopyridine nucleotide (DPN) was observed in the system oxidizing β-hydroxybutyrate. This system was insensitive to chlorpromazine. The succinate system also failed to phosphorylate after pre-incubation in water, and as before was unaffected by chlorpromazine.

In Table 3 are given figures for a somewhat different system, in which cytochrome *c* is reduced by β-hydroxybutyrate. In these experiments, the mitochondria were pre-incubated in 0·075 M-sucrose or water as before, but the reaction medium did not contain the ATP, hexokinase and glucose or Mg^{2+} ions which were added for the measurement of oxidative phosphorylation. It can be seen that chlorpromazine inhibits the electron transport in mitochondria pre-incubated in 0·075 M-sucrose, but not in those suspended in water.

Similar results were obtained with DPNH as substrate. This substrate was used with mitochondria in 0·25 M-sucrose, mitochondria in 0·075 M-sucrose and mitochondria in water. When mitochondria in 0·25 M-sucrose are used, the oxidation of DPNH proceeds by the so-called external pathway, which is non-phosphorylating. The same is true of the particles suspended in water, whereas those in 0·075 M-sucrose can utilize the internal phosphorylating pathway (Lehninger, 1953–54). These results indicate that chlorpromazine inhibits the DPNH–cytochrome *c* reductase of the internal mitochondrial pathway, in a manner reminiscent of that of Amytal (Ernster, 1956). The nature of the external DPNH–cytochrome *c* reductase remains in doubt. One possibility is that microsomal contamination of the mitochondrial preparations is responsible. Distribution studies have shown that most of the total DPNH–cytochrome *c* reductase activity is present in the microsome fraction (Hogeboom & Schneider, 1950). If this were the chlorpromazine-insensitive path, it might mean that in water-treated mitochondria electron transport was switched to a non-mitochondrial path. In Table 4 are shown results with mitochondria washed five times in iso-osmotic sucrose by resuspension and sedimentation at 8000 *g* for 15 min. for each centrifuging. It may be seen that the DPNH–cytochrome *c* reductase activity of the particles declines considerably. The loss in DPNH–cytochrome *c* reductase activity is not due to physical loss of mitochondria, since there was only a slight fall (10 %) in succinoxidase activity during the washing procedure. This decline might be due to leaching of the enzyme from the mitochondria rather than washing out of microsomes. However, the preparation is still sensitive to chlorpromazine and the sensitivity is lost on water treatment. The DPNH–cytochrome *c* reductase activity of isolated microsomes is not inhibited by chlorpromazine. It seems reasonable therefore to assume that the external pathway for the oxidation of DPNH is a genuine mitochondrial-enzyme system, and not simply due to contamination with microsomes.

It may be observed in Table 4 that, where sucrose suspensions were used, mitochondria were

Table 3. *Effect of chlorpromazine on the reduction of cytochrome* c *by β-hydroxybutyrate*

Components of the system were: β-hydroxybutyrate, 33 mM; cytochrome *c*, 40 μM; inorganic sodium orthophosphate buffer, pH 7·2, 33 mM; NaCN, 0·1 mM; ethylenediaminetetra-acetic acid, neutralized to pH 7·2 with NaOH, mM; mitochondria (0·008 mg. of N) were added in 0·1 ml. of 0·075 M-sucrose or in 0·1 ml. of water. With water suspensions, DPN (1 μmole) was also added. Final volume was 3 ml. Temperature was 20°. The reaction was followed by observing the increase in absorption at 550 mμ in optical cells of 1 cm. light path in the Unicam SP. 500 spectrophotometer. Blanks contained all components other than substrate and the reaction was started by addition of enzyme. Readings were taken at 1 min. intervals thereafter for a period of at least 10 min.

Suspension medium for mitochondria	Concn. of chlorpromazine (mM)	Cytochrome *c* reduced (μmoles/mg. of N/hr.)	Inhibition (%)
0·075 M-Sucrose	—	12·8	Nil
0·075 M-Sucrose	0·1	6·4	50
Water	—	15·6	Nil
Water	0·1	17·6	Nil

Table 4. *Effect of extensive washing on mitochondrial reduced diphosphopyridine nucleotide–cytochrome* c *reductase*

Components of the system were as described in Table 2, except that DPNH, 0·67 mM, replaced β-hydroxybutyrate as substrate. Enzyme additions and other methods were also as described in Table 2. The washing procedure for the mitochondria was as described in the text.

Suspension medium for mitochondria	No. of washes in 0·25 M-sucrose	Cytochrome reduced (μmoles/mg. of N/hr.) Control	With 0·1 mM-chlorpromazine	Inhibition (%)
0·25 M-Sucrose	1	45·0	45·0	Nil
Water	1	42·5	45·0	Nil
0·25 M-Sucrose	5	12·0	5·0	59
Water	5	8·8	9·6	Nil

added in 0·25 M-sucrose. The reason for this is that repeated sedimentation and resuspension, even in the presence of mM-EDTA, causes mitochondrial damage and an increase in mitochondrial permeability. This is probably due both to ageing (Christie & Judah, 1954) and to mechanical disruption. This is inferred from the fact that in parallel experiments when these mitochondria were suspended in 0·075 M-sucrose the addition of DPN was necessary before reduction of cytochrome *c* could be obtained by β-hydroxybutyrate.

It may be concluded that chlorpromazine inhibits mitochondrial DPNH–cytochrome *c* reductase. Disruption of the particles removes the inhibition. This release of inhibition might happen in two ways: (1) chlorpromazine acts primarily on a system associated with the electron-transport chain but not part of it, e.g. the oxidative-phosphorylation mechanism. Disruption of the mitochondria in this way would then lead to dissociation of the two systems, and the effect of chlorpromazine on the respiratory chain would vanish. (2) Chlorpromazine acts on a component of the respiratory chain which is bypassed in disrupted mitochondria. For example, cytochrome *b* could be the sensitive component of the respiratory chain, since Chance & Williams (1956) have suggested that it no longer takes part in the non-phosphorylating electron transport which occurs upon water-disruption of mitochondria.

We investigated these two possibilities of chlorpromazine action. In the section which follows, the effect of chlorpromazine on succinate oxidation is described. Cytochrome *b* is a component of this system in both phosphorylating and non-phosphorylating forms (Chance & Williams, 1956).

Inhibition of succinate oxidation

In our previous experiments (Dawkins *et al.* 1959) we had observed that the oxidation of succinate appeared to be relatively insensitive to chlorpromazine. In the system used in these experiments, succinate oxidation does not proceed at the maximal rate and can be considerably increased by increasing the phosphate and cytochrome *c* concentrations. Under conditions where maximal rates of oxidation are possible chlorpromazine is found to be inhibitory, both in intact mitochondria and in particles disrupted with water, although somewhat less so in the latter (Table 5).

We therefore tested the effect of chlorpromazine on the reduction of cytochrome *c* by succinate, and have shown (see Table 2) that it was not inhibitory at a concentration of 0·2 mM, whether the mitochondria were suspended in water or 0·075 M-sucrose.

It may be concluded that chlorpromazine cannot act on a component common to the DPNH–cytochrome *c* reductase system and the span between succinate and cytochrome *c*. The inhibition of the succinoxidase system by chlorpromazine therefore depends solely on its inhibitory action towards the oxidation of ferrocytochrome *c* (Dawkins *et al.* 1959).

Table 5. *Inhibition of succinoxidase by chlorpromazine*

Components of the system were: cytochrome *c*, 0·4 mM; inorganic sodium orthophosphate buffer, pH 7·2, 85 mM; EDTA, mM; succinate, 33 mM. Mitochondria (0·04 mg. of N) were added in 0·5 ml. of 0·25 M-sucrose or in 0·5 ml. of water, as indicated. Final volume in the flasks was 3 ml. The centre well contained 0·1 ml. of 30% (w/v) KOH and filter paper. Temperature was 38°. Manometric measurements were started after 7 min. thermal equilibration and continued for 60 min. thereafter.

Suspension medium for mitochondria	Concn. of chlorpromazine (mM)	Uptake of O_2 (μg.atoms)	Inhibition (%)
0·25 M-Sucrose	—	6·4	Nil
	0·1	2·0	70
Water	—	5·3	Nil
	0·1	2·3	54

In the following sections are described experiments which were designed to test the hypothesis that the action of chlorpromazine is on the coupled phosphorylation mechanism.

Action of chlorpromazine on the ^{32}P-adenosine triphosphate-exchange reaction

We have shown in our previous paper (Dawkins *et al.* 1959) that chlorpromazine has no effect on the efficiency of the phosphorylation coupled to the reduction of cytochrome *c* by β-hydroxybutyrate and L-glutamate, and it was therefore of interest to see whether it would influence the ^{32}P–ATP-exchange reaction described by Boyer, Falcone & Harrison (1954). These authors ascribed the exchange to a reversal of oxidative phosphorylation, presumably due to electron flux in the steady-state conditions of the mitochondria.

This experiment of necessity has to be conducted in intact mitochondria, since it is known that mitochondrial damage will reduce the exchange (Spector & Judah, unpublished work).

Table 6 shows that, at 0·2 mM, chlorpromazine brings about a 50% inhibition of the exchange. Under the conditions of this experiment, adenosine triphosphatase action is negligible, no more than 5% of ATP phosphorus being set free during the incubation.

This result is of some interest, since it has been shown that antimycin A, which inhibits electron

transport between cytochrome *b* and *c* (Chance & Williams, 1956), has no effect on the exchange reaction (Judah & Spector, unpublished work).

This effect of chlorpromazine may mean either that the exchange reaction is due largely to electron flux in a limited part of the respiratory chain or that the inhibition by chlorpromazine is due to interference with some factor as yet unknown. This factor may well be related to the oxidative-phosphorylation system, inhibition of which would lead to inhibition of respiration in a phosphorylating system but not in a non-phosphorylating mitochondrial system.

Under these circumstances we felt it would be of interest to see whether 2:4-dinitrophenol can influence the respiratory inhibition caused by chlorpromazine.

Action of chlorpromazine on respiration stimulated by 2:4-dinitrophenol

The manometric experiments so far cited in this paper were performed in a system designed to give respiratory rates which were not limited by the availability of adenosine diphosphate (ADP) (see Chance & Williams, 1956). Table 7 shows an experiment in which the effect of chlorpromazine on respiration is shown in a system without added phosphate acceptor. The rate of oxidation of this system is limited by availability of ADP. This system is compared with others in which hexokinase and glucose are added, and one in which dinitrophenol is added. Dinitrophenol under these conditions will stimulate respiration by making the system independent of ADP concentration (Lardy & Wellman, 1952).

It will be observed that whereas chlorpromazine has little effect on the system which is rate-limited by ADP concentration, it prevents the stimulation of respiration brought about by hexokinase and glucose or by dinitrophenol. This experiment supports the view that chlorpromazine acts upon a phosphorylating process which is closely coupled to the electron-transport chain, and the site of action is at a point before that of dinitrophenol.

Further support for this view is given by the experiments shown in Table 8. In these, chlorpromazine was tested in a system deficient in inorganic phosphate and in which respiration is slow. Contrary to the results obtained with ADP-deficient systems, there was a definite stimulation of respiration. That this was not maximal is shown by the effect of dinitrophenol, which accelerated oxygen uptake about sixfold. In keeping with the hypothesis given above, this extra oxygen uptake was markedly inhibited by chlorpromazine. It may be inferred, also, that the stimulation of respiration by chlorpromazine is due to limited breakdown of some phosphate-containing compound. That it is

Table 6. *Effect of chlorpromazine on the ^{32}P–adenosine triphosphate-exchange reaction*

Components of the system were: ATP, 2·5 mM; inorganic sodium orthophosphate buffer, pH 7·2, 5 mM; KCl, 25 mM; $MgSO_4$, 5 mM. ^{32}P equivalent to 15 000 counts/min. was added to each vessel. For the measurement of radioactivity a liquid counter was used. Measurements were corrected for background counts, isotope decay and dead time of the instrument. Mitochondria (1 mg. of N) were added in 0·5 ml. of 0·25 M-sucrose. Final volume was 2 ml. and the temperature was 20°. Duration of the experiments was 15 min.

		Incorporation of ^{32}P into ATP (counts/min.)	Inhibition (%)
Control		3150	Nil
Chlorpromazine	(0·1 mM)	3000	5
	(0·2 mM)	1460	50

Table 7. *Effect of 2:4-dinitrophenol and chlorpromazine on L-glutamate oxidation*

Components of the system were as described in Table 1, with the following exceptions: L-glutamate, 10 mM, was used as substrate; hexokinase and glucose were omitted unless indicated in the Table; mitochondria equivalent to 1 mg. of N were added in 0·5 ml. of 0·25 M-sucrose. Final volume was 3 ml. Temperature was 38°; the duration of the experiment was 10 min., after 7 min. thermal equilibration.

Additions	Concn. of chlorpromazine (mM)	Uptake of O_2 (μg.atoms)
Nil	Nil	4·5
Nil	0·1	4·0
Nil	0·2	3·6
Glucose (30 mM) + hexokinase	Nil	10·0
Glucose (30 mM) + hexokinase	0·2	4·0
2:4-Dinitrophenol (33 μM)	Nil	8·1
2:4-Dinitrophenol (33 μM)	0·1	5·0
2:4-Dinitrophenol (33 μM)	0·2	3·1

Table 8. *Effect of chlorpromazine on respiration in phosphate-deficient systems*

Components of the system were: ATP, 1·7 mM; KCl, 25 mM; $MgSO_4$, 6·7 mM; L-glutamate, 10 mM; cytochrome *c*, 20 μM; inorganic sodium orthophosphate buffer, pH 7·2, 1·7 mM; 2-amino-2-hydroxymethylpropane-1:3-diol-HCl buffer, pH 7·2, 33 mM. Mitochondria (0·4 mg. of N) were added in 0·5 ml. of 0·25 M-sucrose. Final volume was 3 ml. There was filter paper in the centre well with 0·1 ml. of 30% (w/v) KOH. Temperature was 38°. Manometric readings were started after 7 min. thermal equilibration and continued for 10 min. thereafter.

Additions	Uptake of O_2 (μg.atoms)
Nil	1·1
Chlorpromazine (0·2 mM)	2·1
2:4-Dinitrophenol (33 μM)	6·0
Chlorpromazine (0·2 mM) + 2:4-dinitrophenol (33 μM)	3·6

Table 9. *Effect of Amytal on oxidative phosphorylation with succinate and ferrocytochrome* c *as substrates*

For the study of phosphorylation coupled to oxidation of ferrocytochrome *c* the components were as described in Table 1 except that the substrate was L-ascorbic acid (neutralized to pH 7·2 with NaOH just before use), 10 mM. Mitochondria (2 mg. of N) were added in 0·5 ml. of 0·075 M-sucrose. Final volume was 3 ml. The temperature was 20° and the manometric readings were continued for 60 min. after 7 min. thermal equilibration. For the study of phosphorylation coupled to oxidation of succinate, the components were as described in Table 1 except that sodium succinate, 33 mM, was substrate. Mitochondria (0·2 mg. of N) were added in 0·5 ml. of 0·25 M-sucrose. Final volume was 3 ml. and the temperature was 38°. Thermal equilibration was 7 min. and readings were continued for 30 min. thereafter.

Substrate	Concn. of Amytal	Uptake of O_2 (μg.atoms)	Uptake of P (μmoles)	P/O	Inhibition of P/O (%)
Ferrocytochrome *c*	—	10·9	6·5	0·6	Nil
Ferrocytochrome *c*	2 mM	9·6	2·4	0·25	38
Succinate	—	7·3	9·7	1·33	Nil
Succinate	2 mM	6·2	5·3	0·80	40

Table 10. *Effect of Amytal on the* ^{32}P–*adenosine triphosphate-exchange reaction*

Components of the system and other details of this experiment were as described in Table 5.

	Incorporation of ^{32}P into ATP (counts/min.)	Inhibition (%)
Control	3150	Nil
Amytal (2 mM)	1890	60

not due to stimulation of ATP breakdown was shown by analysis of inorganic phosphate before and after incubation, which revealed no change in level of inorganic phosphate in the medium at the end of the experiment.

There was a slight possibility that this stimulation of respiration by chlorpromazine was due to the uncoupling effect, demonstrated previously (see Dawkins *et al.* 1959). This was excluded by two experiments. In one, the anaerobic reduction of cytochrome *c* by β-hydroxybutyrate was studied. Chlorpromazine stimulated this reaction in phosphate-deficient media. In the second, the aerobic oxidation of L-glutamate was stimulated in phosphate-deficient systems by a concentration of chlorpromazine having no uncoupling action (60 μM).

Observations on Amytal

Since our results with chlorpromazine suggest an action similar to that of Amytal described by Ernster (1956), we studied a few properties of the latter. Amytal at a concentration of 2 mM was shown, in agreement with Ernster, to inhibit glutamate and β-hydroxybutyrate oxidation by some 80 %. On succinate oxidation at this concentration it was only slightly inhibitory and we therefore tested its action on oxidative phosphorylation with this substrate and with ferrocytochrome *c*.

Table 9 shows that Amytal inhibits partially the oxidative phosphorylation with both these substrates, and that it does not therefore exhibit any clear-cut separation of effects as does chlorpromazine. Dawkins *et al.* (1959) have shown that chlorpromazine inhibits the phosphorylation coupled to oxidation of ferrocytochrome *c* at a concentration which does not affect phosphorylations coupled to the other steps of the electron-transport chain.

Table 10 shows that Amytal at a concentration of 2 mM inhibits the ^{32}P–ATP exchange by some 60 %, so that in this respect, too, it resembles chlorpromazine.

DISCUSSION

The evidence presented in this paper shows that chlorpromazine inhibits electron transport between DPNH and cytochrome *c* in phosphorylating systems. No inhibition is found in non-phosphorylating systems, i.e. in mitochondria disrupted with water. Previously we have shown that even at the inhibited rate in the presence of chlorpromazine the phosphorylating efficiency for this span is normal (Dawkins *et al.* 1959). Since chlorpromazine does not affect the span succinate to cytochrome *c* in any system, it follows that none of the components common in both spans is involved. It also follows that chlorpromazine must act between DPNH and flavoprotein.

The observation that chlorpromazine affects only phosphorylating systems suggests the possibility that its action is not directly on the respiratory carriers, but on some intimately coupled system.

We suggest that chlorpromazine acts on some component of the normal phosphorylating mechanisms. The evidence can be summarized as follows:

(1) Chlorpromazine inhibits electron transport between DPNH and cytochrome *c* without altering the phosphorylating efficiency for the span.

(2) It has little effect on the respiration of DPN-linked substrates which are limited by a low concentration of ADP.

(3) It prevents the stimulation of respiration in ADP-deficient systems brought about by 2:4-dinitrophenol or a phosphate-acceptor system.

(4) It is able to stimulate respiration in a phosphate-deficient system without liberating inorganic phosphate.

(5) It inhibits the ^{32}P–ATP-exchange reaction.

The minimum interpretation of these findings is that chlorpromazine and dinitrophenol act at different points in the sequence of phosphorylation reactions. It would seem that chlorpromazine prevents the formation of the intermediate which is hydrolysed under the influence of dinitrophenol (Chance & Williams, 1956; Lehninger, 1958). This indicates the existence of at least two intermediates in the series of phosphorylation reactions, which can be formulated into the following sequence:

$$AH_2 + B + P_i \rightleftharpoons BH_2 + A \sim P \quad (1)$$

$$A \sim P + X \rightleftharpoons X \sim P + A \quad (2)$$

$$X \sim P + ADP \rightleftharpoons ATP + X \quad (3)$$

where A and B are respiratory carriers and X is an unknown intermediate. It is postulated that chlorpromazine inhibits reaction (2) and that dinitrophenol hydrolyses the compound X ~ P.

We believe that this scheme explains our findings by a single hypothesis. Considering the effects of chlorpromazine on electron transport, the scheme shows that in systems respiring freely with an active coupled phosphorylation, reaction (2) becomes rate-limiting in the presence of chlorpromazine. The rate of respiration is reduced, but there is no diminution in the efficiency of the phosphorylation. If inorganic phosphate is lacking, reaction (1) must limit the rate of respiration. In the presence of chlorpromazine, the compound A ~ P would become available for hydrolysis, since reaction (2) is inhibited, and this would permit some stimulation of respiration by chlorpromazine in phosphate-deficient systems. The breakdown of A ~ P in the presence of chlorpromazine is necessarily slow, since otherwise chlorpromazine would have an uncoupling action on oxidative phosphorylation without inhibiting respiration. It is clear, also, that when reaction (3) is limiting (ADP-deficient systems) chlorpromazine can have no effect on the respiratory activity of the system.

The scheme also explains why chlorpromazine prevents the stimulatory effect of dinitrophenol on phosphate- and ADP-deficient systems.

Recently, Lehninger (1958) claims to have isolated a phosphate-transferring enzyme catalysing a reaction of the same type as reaction (3). This enzyme is insensitive to dinitrophenol, and Lehninger suggests that it is the terminal step in oxidative phosphorylation. If this is so, then some preceding step must be sensitive to dinitrophenol. From our findings, any formulation which accounts for all the facts must include yet another intermediate.

Lehninger's scheme is as follows:

$$AH_2 + B + X \rightleftharpoons A \sim X + BH_2 \quad (4)$$

$$A \sim X + P_i \rightleftharpoons X \sim P + A \quad (5)$$

$$X \sim P + ADP \rightleftharpoons ATP + X \quad (6)$$

in which A and B are respiratory carriers and X is an unknown intermediate. A ~ X is hydrolysed by dinitrophenol. Clearly, this scheme does not allow for a separate site of action for chlorpromazine. Furthermore, it must mean that dinitrophenol makes the system independent of phosphate concentration, which is not the case. Extensive investigation of this point (Judah, 1951) has shown that the stimulatory effect of dinitrophenol is not observed when the concentration of inorganic phosphate is below a certain critical level. Therefore any intermediate hydrolysed by dinitrophenol must contain inorganic phosphate. In our scheme we have followed the example of Lehninger (1958) in formulating the oxidized respiratory carrier as the 'energy-rich' form, whereas in a previous communication the opposite was inferred from the scheme of Chance & Williams (1956). This makes little difference to the hypothesis about the site of action of chlorpromazine.

The results with the ^{32}P–ATP-exchange reaction support our hypothesis that chlorpromazine inhibits reaction (2). Boyer *et al.* (1954) suggest that this exchange is due to reversal of oxidative phosphorylation. But Chance & Williams (1956) point out that there is a possibility that intermediates (e.g. X ~ I in their scheme) could bring ^{32}P and ATP into equilibrium. The latter view is supported by our results, since under the conditions of the exchange reaction (i.e. low ADP, high ATP) chlorpromazine has been shown not to inhibit electron flow. In a previous paper (Dawkins *et al.* 1959) we have shown that the effect of chlorpromazine on oxidative phosphorylation is limited to inhibition of the phosphorylation coupled to the terminal step in electron transport (the oxidation of ferrocytochrome *c*). This phosphorylation probably does not play any part in the exchange reaction as neither antimycin A nor cyanide inhibits the exchange reaction. Nevertheless, chlorpromazine inhibits the exchange powerfully. This result is in accord with our interpretation that chlorpromazine interacts with a component of the phosphorylating mechanism coupled to electron flow. It would seem from these results that the major part of the exchange reaction is related to the steps between DPNH and cytochrome *c*.

The results obtained by Ernster (1956) with Amytal show that its action is very similar to that of chlorpromazine in that it inhibits mitochondrial DPNH–cytochrome *c* reductase only in undamaged mitochondria. After the mitochondria are damaged with Ca^{2+} ions Amytal is no longer inhibitory. Our own observations with Amytal show that it also inhibits the exchange reaction but it differs from chlorpromazine in that it uncouples both phosphorylations associated with the oxidation of succinate.

The results of the previous paper (Dawkins *et al.* 1959) indicate that chlorpromazine uncouples the phosphorylation coupled to the oxidation of ferrocytochrome *c* and also inhibits cytochrome oxidase. However, a unified hypothesis for all these actions is not possible, since closely related phenothiazines have no action on cytochrome oxidase but are as effective as chlorpromazine on electron transport between DPNH and cytochrome *c* in a phosphorylating system. Finally, the pharmacological activity must be considered. In the previous paper (Dawkins *et al.* 1959) we suggested that the inhibitory effect of chlorpromazine on cytochrome oxidase might account for its pharmacological activity. We are investigating a series of compounds related to chlorpromazine. At least one of these has little effect on cytochrome oxidase, but is a powerful inhibitor of DPNH–cytochrome *c* reductase in phosphorylating systems. This substance is also a powerful tranquillizer and it seems possible that the inhibition of DPNH–cytochrome *c* reductase is related to tranquillizing activity. It is of interest that the sulphoxide of chlorpromazine has very little activity in our systems. This compound is the detoxication product of chlorpromazine and has no tranquillizing action.

SUMMARY

1. The action of chlorpromazine on the respiratory chain has been investigated. Inhibition of electron transport between reduced diphosphopyridine nucleotide and cytochrome *c* has been demonstrated.

2. This inhibition occurs only in phosphorylating systems and is not observed in succinate–cytochrome *c* reductase systems.

3. The inhibitory action is due to an effect on the coupled phosphorylation reaction rather than that of an interruption in electron transport.

4. Chlorpromazine acts at a point in the phosphorylation reaction other than that affected by 2:4-dinitrophenol.

5. On the basis of these findings a scheme for the reaction involved in oxidative phosphorylation is proposed.

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REFERENCES

Borgstrom, B., Sudduth, H. C. & Lehninger, A. L. (1955). *J. biol. Chem.* **215**, 571.

Boyer, P. D., Falcone, A. B. & Harrison, W. H. (1954). *Nature, Lond.*, **174**, 401.

Chance, B. & Williams, G. R. (1956). *Advanc. Enzymol.* **17**, 65.

Christie, G. S. & Judah, J. D. (1954). *Proc. Roy. Soc.* B, **142**, 241.

Dawkins, M. J. R., Judah, J. D. & Rees, K. R. (1958). *Nature, Lond.*, **182**, 875.

Dawkins, M. J. R., Judah, J. D. & Rees, K. R. (1959). *Biochem. J.* **72**, 204.

Ernster, L. (1956). *Exp. Cell Res.* **10**, 721.

Hogeboom, G. H. & Schneider, W. C. (1950). *J. nat. Cancer Inst.* **10**, 983.

Judah, J. D. (1951). *Biochem. J.* **49**, 271.

Judah, J. D. & Williams-Ashman, H. G. (1951). *Biochem. J.* **48**, 33.

Kennedy, E. P. (1953). *J. biol. Chem.* **201**, 399.

Lardy, H. A. & Wellman, H. (1952). *J. biol. Chem.* **195**, 215.

Lehninger, A. L. (1953–54). *Harvey Lect.* **49**, 176.

Lehninger, A. L. (1958). *Science*, **128**, 450.

Nielsen, S. O. & Lehninger, A. L. (1955). *J. biol. Chem.* **215**, 555.

Incorporation of DL-[1-^{14}C]Leucine and [1-^{14}C]*iso*Valeric Acid into Milk Constituents by the Perfused Cow's Udder

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The older view on the composition of animal fats—that the only naturally occurring saturated fatty acids are those possessing an even number of carbon atoms—has in recent years been shown to be incorrect. Shorland and his co-workers in New Zealand (see Shorland, 1956) have in a series of papers demonstrated the occurrence in a number of fats of both branched and straight-chain odd-numbered acids. Similar results were obtained by James & Martin (1956) in the development of the gas–liquid chromatogram for the separation of the long-chain fatty acids, particularly large amounts of such branched and odd-numbered acids being found in the milk of ruminants.

The even-numbered acids have been shown to be synthesized in the udder of lactating animals by condensation of acetate units (Popják, French & Folley, 1951; Popják, French, Hunter & Martin, 1951; Cowie *et al.* 1951). The odd-numbered straight-chain acids, however, were found to be formed by the condensation of propionate with acetate in the isolated perfused cow's udder (James, Peeters & Lauryssens, 1956). The demonstration by El-Shazly (1952*a*) that the micro-organisms of sheep rumen were capable of deaminating amino acids to give the C_4 and C_5 branched-chain acids (*iso*butyric acid, *iso*valeric acid and methylethylacetic acid) suggested that some of these acids might be precursors of the long-chain *iso* and *anteiso* odd-numbered acids (El-Shazly, 1952*b*).

In order to investigate this point we have studied the utilization of both DL-[1-^{14}C]leucine and [1-^{14}C]*iso*valeric acid by the isolated perfused cow's udder, for synthesis of fatty acids, proteins and the Krebs-cycle acids.

EXPERIMENTAL

Udder perfusions. The two experiments were carried out on perfused lactating half-udders obtained from two different cows, by using the method described by Peeters & Massart (1952). Tests for chronic mastitis, with both clinical examination and the Hotis test (Hotis & Miller, 1936), were negative. Immediately before slaughter the cows were milked out with the aid of intravenously injected oxytocin. The udders were removed and perfused for 2 min. with Ringer–Tyrode solution to remove blood, bisected along the median septum and connected to the perfusion apparatus. The teats were cut off at the top and cannulae were inserted; the residual milk was allowed to drain and was discarded. Perfusion was carried out with 8 l. of fresh heparinized cow's blood, the radioactive substrates being added to the artificial lung 30 min. after the perfusion was started. The following substrates were used: DL-[1-^{14}C]leucine (0·95 mc, 23·37 mg.) in 50 ml. of water (after addition of a few drops of N-NaOH) and sodium [1-^{14}C]*iso*valerate (0·5 mc, 62·06 mg.) in 50 ml. of water. After addition of the labelled materials, a solution of inactive sodium acetate (10·5 g. in 500 ml.) was added to the blood reservoir throughout the perfusion from a constant-drip device.

The gas evolved from the artificial lung was aspirated through NaOH solution to remove CO_2, the solution being replaced every 15 min. Approximately 80% of the evolved CO_2 was collected. The experiments were carried out under a large hood secured with a plastic curtain to prevent escape of labelled CO_2 into the atmosphere.

After 150 min. milk was released by injection of oxytocin (10 i.u.) into the udder artery, 160 ml. of milk being obtained in the leucine experiment and 140 ml. in the *iso*valerate experiment. The skin, teats and adipose tissue were removed by careful dissection and the residual udder tissue was cut into pieces and stored at $-15°$ for 1 hr.

Extraction of fat. The milk fat was removed by centrifuging and dissolved in ether. The udder tissue was minced and extracted for several days with ethanol–ether (3:1, v/v) in a Soxhlet apparatus. The ethanol–ether was distilled off and the fat extracted with ether or light petroleum, insoluble material being discarded. From each kilogramme of wet tissue 30–50 g. of crude fat was obtained. Traces of labelled *iso*valeric acid were removed by washing the ether extracts several times first with 1% sodium bicarbonate and then with water. The ether layers were concentrated to small volume and the phospholipids were precipitated by adding acetone and a few drops of ethanolic $MgCl_2$. The neutral fat was saponified with ethanolic KOH on the water bath for several hours, water was added and the ethanol distilled off. Cholesterol was extracted with ether and precipitated as the digitonide. The aqueous layer was acidified with H_2SO_4 and the lower fatty acids were removed by steam-distillation; the long-chain acids were extracted with ether. Glycerol was extracted from the aqueous residue by refluxing with successive portions of ethyl acetate and precipitated as the trinitrobenzoate (Popják, Glascock & Folley, 1952).

Separations of the fatty acids. The steam-volatile fatty acid fractions (80–160 mg.) were dissolved in 35% (v/v) acetone in water and applied to the reversed-phase liquid–liquid column (Howard & Martin, 1950), containing 40 g. of Hyflo Super-Cel. The first zone eluted consisted of a mixture of acetic acid, butyric acid and caproic acid, the higher even-numbered acids being obtained as single peaks (C_8–C_{14}). The sodium salts from the first zone were acidified and the free acids were extracted with ether and dried over anhydrous Na_2SO_4. The ether was removed and the mixture of acetic acid, butyric acid and caproic acid was separated in 2 mg. amounts on the gas–liquid chromatogram (James & Martin, 1952).

The non-volatile long-chain fatty acid fraction (C_{14}–C_{18}) was separated initially on the reversed-phase liquid–liquid column. Each peak emerging was acidified, dissolved in ethyl acetate and hydrogenated over palladinized charcoal to a zero iodine value. Each peak was then rechromatographed. In this way all the even-numbered saturated and unsaturated acids from C_2 to C_{18} were separated. The resolving power of these liquid–liquid columns was insufficient to separate the odd-numbered straight- and branched-chain acids, so this was done with the gas–liquid chromatogram described by James & Martin (1956) and with the gas-density meter as detector (Martin & James, 1956). Fractions were collected as described by James *et al.* (1956).

Analytical methods. Total cholesterol was estimated according to Kenny (1952), iodine values by the semi-micro method of Trappe (1938) and glycerol by the Lambert & Neish (1950) technique.

Isolation of milk proteins. The skimmed milk was adjusted to pH 4·5, to precipitate the casein, which was redissolved in water, after centrifuging, by dropwise addition of N-NaOH to pH 7·0. This isoelectric precipitation and re-solution was repeated three times. Perchloric acid (6N) solution was added to the whey to give a final concentration of 0·35N. After standing overnight in the refrigerator the precipitate was removed by centrifuging and redissolved in water by addition of a few drops of N-NaOH. Addition of an equal volume of 20% trichloroacetic acid precipitated the whey proteins; these were washed three times with ethanol, then with ether and dried at 40°.

Amino acid separations. The casein samples were refluxed with 6N-HCl for 36 hr. Excess of HCl was removed by repeated evaporation *in vacuo*, the last traces being removed by leaving the hydrolysate overnight in a vacuum desiccator over soda lime. The residue was dissolved in water and the insoluble material deposited after being kept overnight at +2° was discarded. The pH was then adjusted to between 3·5 and 4·5 by dropwise addition of 0·1N-NaOH, and the solution was applied to a Dowex 1 column and eluted with 0·5N-acetic acid. In this way the acidic amino acids and tyrosine were separated, the neutral and basic amino acids composing the first zone eluted being resolved by a Dowex 50 column (Hirs, Moore & Stein, 1954). The mixed zones of serine and threonine and of isoleucine and methionine were separated on a starch column (Moore & Stein, 1949), being eluted with propan-1-ol–0·5N-HCl. All zones were located by the colorimetric procedure of Cocking & Yemm (1954), with a mixture of ninhydrin and KCN. Any contaminants introduced in the column procedures were removed by passing the amino acid solutions through small Dowex 50 columns, HCl being used for elution. The purity and identity of the separated amino acids was checked by paper chromatography. The concentration of the amino acids in solution may be accurately determined by colorimetry, with the method of Cocking & Yemm (1954), or by the micro-Kjeldahl method with micro-diffusion techniques (Conway, 1950).

Isolation of some acids of the Krebs cycle. After extraction of casein and the whey proteins from the skimmed milk, the filtrate was adjusted to pH 3·0 with 5N-KOH. After storage at 2° for 24 hr. the precipitated $KClO_4$ was filtered off.

Udder tissue (500 g.) was homogenized at 2° in 2 vol. of iced water with addition of perchloric acid to a final concentration of 0·35N. The mixture was stored at 2° for 24 hr., the proteins were removed by centrifuging and resuspended in 0·35N-perchloric acid and centrifuged. The combined supernatants contained, in addition to the Krebs-cycle acids, a strongly acid viscous substance that interfered with the subsequent chromatographic procedures. This material was removed as follows: 5N-KOH was added to give a final pH of 3·0 and the mixture was stored at +2° for 24 hr. The precipitated $KClO_4$ was filtered off, the supernatant was concentrated *in vacuo* at 40°, filtered and passed through a Dowex 50 H^+ column. The acid solution emerging was treated with 10 vol. of ethanol and stored at 2° for several days; the resulting precipitate was filtered off, the filtrate neutralized with NaOH and evaporated to dryness *in vacuo*. The residue was dissolved in water and filtered.

Both milk and tissue extracts were passed through a Dowex 1 $H{\cdot}CO_3^-$ column (30 cm. × 3 cm.) (Busch, Hurlbert & Potter, 1952). The column was washed with CO_2-free water and gradient-elution was then done with formic acid ranging from 0 to 7N; 15 ml. fractions were collected. Lactic acid emerged as soon as the eluent became acid and was detected colorimetrically (Mendel & Goldscheider, 1925). Citric acid was detected (Natelson, Pincus & Lugovoy, 1948) in the fractions containing 2 to 3N-formic acid. The citric acid fractions were combined, evaporated, dissolved in a minimal volume of 35% (v/v) butanol–$CHCl_3$ and applied to a silica-gel column (Isherwood, 1946). The fractions eluted from the column were titrated with 0·1N-NaOH. In the analyses of the udder-tissue extracts, material eluted from the Dowex 1 column between lactic acid and citric acid was pooled and evaporated. The mixture was dissolved in a minimal volume of 35% (v/v) butanol–$CHCl_3$ and run on a silica-gel column. On elution with 10% (v/v) butanol–$CHCl_3$ two small peaks were produced containing succinic acid, malic acid and two unidentified acids. These substances were separated by paper chromatography with *iso*amyl formate–water–formic acid (11:1:2, by vol.), as described by Kalbe (1954). The mixture was applied as a streak and the zones were located with methyl red (pH 8·0); four well-defined zones were obtained, the fastest being unidentified, the second succinic acid, followed by another unidentified substance and then malic acid. The separated zones were eluted with water and further purified on a paper chromatogram with a phenol–xylol–85% formic acid (5:5:2, w/w/v) system. The zones were eluted with water, evaporated *in vacuo* over P_2O_5 and redissolved in 0·5 ml. of water. Malic acid and succinic acid were estimated on a one-dimension chromatogram by measurement of spot area with a planimeter, a series of standards of known concentration being used (Block, Durrum & Zweig, 1955). An accuracy of approximately 10% was obtained.

Radioactive assays. Most measurements were carried out with a windowless flow counter. The soaps were dissolved in a minimum amount of 50% ethanol and plated on plastic disks of 2 cm.², of the type described by Popják (1950). The samples were dried under an infrared lamp and weighed: usually amounts of 2–4 mg. were plated. An accurately known volume of the concentrated amino acid solution containing from 20 to 1000 μg. of carbon was pipetted on the disks. Organic acids were dissolved in water and plated in the same manner. The dried samples were assayed for radioactivity. Lactose, glyceryl trinitrobenzoate, phospholipids, cholesterol digitonide and proteins were oxidized to CO_2 by the wet combustion method (Lindenbaum, Schubert & Armstrong, 1948) and the $^{14}CO_2$ was counted as $BaCO_3$. All counts were corrected for dead time of the instrument and background (32–39 counts/min.). Self-absorption corrections were made with the aid of experimentally determined curves (for soaps, amino acids, organic acids and $BaCO_3$). In the counting system used, 1 μmc corresponded to 660 counts/min. above background. The limit which could be detected was 0·003 μmc. The measurements of radioactivity were based on at least 6000 counts, giving a S.E. on the counting rate of 2% for samples with activity not less than 300 counts/min. (0·45 μmc). In the tables we have recorded the activities as μmc, together with the S.D. from the mean from at least triplicate samples. The long-chain fatty acid methyl esters were counted with an end-window counter as previously described (James *et al.* 1956).

RESULTS

Utilization of [1-^{14}C]*leucine*

Carbon dioxide. The low catabolic activity of the leucine was demonstrated by the small amount of $^{14}CO_2$ liberated from the artificial lung. Only 1% of the added dose could be accounted for as CO_2. Production of the labelled CO_2 began slowly, increased to a maximum after 60–75 min. and then decreased up to the end of the experiment (see Fig. 1).

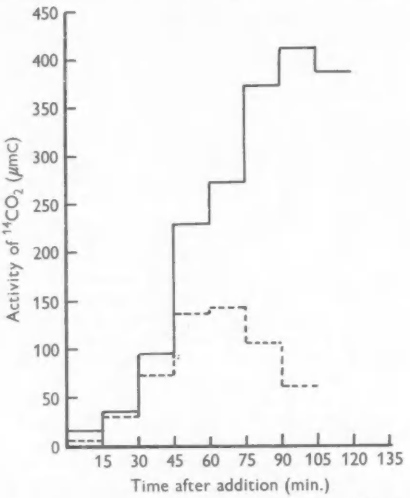

Fig. 1. Activity (μmc) of respiratory CO_2 after addition to the perfusion blood of —, [1-^{14}C]*iso*valeric acid and - - - -, [1-^{14}C]leucine.

Protein. High activity was found in the milk proteins (Table 1). It can be calculated from the specific activities measured in the amino acids that most of the activity (99%) was due to leucine (Table 4). Low activity was detected in glycine and alanine and none in the other amino acids. The specific activity of the isolated casein was six times that found in the *iso*valerate experiment. The total activity of milk casein accounted only for 0·78% of the added ^{14}C.

Table 1. *Specific activities of amino acids isolated from casein in the experiment with* [1-^{14}C]*leucine*

For details see Experimental section.

Isolated substance (milk)	Specific activity (μmc/mg. of C)
Casein	5·10±0·06
Whey proteins	4·73±0·09
Leucine	52·0±0·5
Glycine	0·080±0·006
Alanine	0·010±0·001
Serine	Trace
Valine	Trace

Table 2. *Specific activities of constituents of udder tissue and milk in the experiment with* [1-^{14}C]*isovalerate*

For details see Experimental section.
n.d., not determined.

Substance	Specific activity (μmc/mg. of C) Udder tissue	Milk
Glyceride fatty acids		
Steam-volatile		
Water-soluble	2·30±0·07	—
Water-insoluble	4·8±0·2	0·020±0·002
Non-volatile	0·75±0·06	0·010±0·002
Glycerol	0·00	0·00
Cholesterol	0·020±0·001	n.d.
Phospholipids	0·55±0·02	n.d.
Lactose	n.d.	0·050±0·002
Casein	n.d.	0·87±0·02

Table 3. *Specific activities of amino acids isolated from casein in the experiment with* [1-^{14}C]*isovalerate*

For details see Experimental section. n.d., not determined.

Amino acid	Specific activity (μmc/mg. of C)
Glutamic acid	4·42±0·04
Aspartic acid	1·59±0·02
Serine	0·190±0·003
Glycine	0·038±0·002
Alanine	0·030±0·003
Proline	0·030±0·003
Valine	0·018±0·002

Table 4. *Recovery of* ^{14}C *in the amino acids of total milk casein*

—, no measurable activity.

Substance	[1-^{14}C]Leucine experiment		[1-^{14}C]*iso*Valeric acid experiment	
	Total activity (μmc)	Percentage of total casein activity	Total activity (μmc)	Percentage of total casein activity
Casein	7818±94	—	1013±23	—
Glutamic acid	—	—	929±9	89·3±0·9
Aspartic acid	—	—	94±1	9·0±0·1
Serine	—	—	9·40±0·15	0·90±0·01
Glycine	2·10±0·15	0·030±0·002	0·80±0·05	0·080±0·005
Alanine	0·40±0·04	0·0060±0·0006	0·8±0·1	0·080±0·001
Proline	—	—	4·1±0·4	0·390±0·004
Valine	—	—	1·7±0·2	0·160±0·002
Leucine	7886±79	99·9±1·0	—	—

Fat. No activity could be detected either in the crude fatty acid mixtures obtained from the milk triglycerides or in the acids separated in the reversed phase column. The phospholipids and the lactose were also inactive.

Utilization of [1-^{14}C]iso*valerate*

Carbon dioxide. 5·5% of the administered activity was recovered as CO_2, compared with 1% for leucine, although the time-activity curve had the same general shape; the maximum was reached at a later time (90 min.) (see Fig. 1).

Protein. Table 2 shows that casein is the most active of the protein, lactose and fat fractions isolated from milk. In this case labelling is found in all the non-essential amino acids, with glutamic acid and aspartic acid having the highest activity (Table 3). Little activity could be detected in serine, glycine, alanine and proline, and the essential amino acids were inactive. Of the added ^{14}C, 0·2% was found in milk casein, aspartic acid and glutamic acid being responsible for 98% of the labelling (Table 4).

Fatty acids. Much of the *iso*valeric acid is utilized in the synthesis of the triglyceride fatty acids, the udder-tissue acids having higher activities (by a factor of 100) than the milk acids. The specific activities of the individual acids are shown in Table 5. The specific activity of the lower fatty acids increases with increasing chain length, to reach a maximum at C_{10}, and falls progressively with further increase in chain length. Both udder and milk fatty acids give the same distribution pattern. Some quantitative analyses of the udder and milk lipids are given in Table 6, from which it can be seen that the two samples have, in general, similar compositions. In Table 7 the activities of the individual acids are expressed as a percentage of total fat activity. Separation of the branched- and straight-chain odd- and even-numbered acids by gas–liquid chromatography allowed a determination of their relative specific activities (see

Table 5. *Specific activities of even-numbered acids from udder tissue and milk in the experiment with* [1-^{14}C]iso*valerate*

n.d., not determined.

Acid	Specific activity (μmc/mg. of C)	
	Udder tissue	Milk
Butyric	2·05±0·03	0·00
Caproic (hexanoic)	2·83±0·05	0·00
Caprylic (octanoic)	5·41±0·05	0·06±0·01
Capric (decanoic)	9·42±0·09	0·070±0·004
Lauric	8·49±0·08	0·06±0·01
Myristic	4·41±0·08	(Lost)
Palmitic	1·08±0·02	0·020±0·001
Stearic	0·030±0·005	0·00
Palmitoleic	0·030±0·002	0·00
Oleic	0·040±0·002	0·00
Citric	13·7±0·1	16·8±0·1
Succinic	38·2±3·8	n.d.
Malic	26·9±2·7	n.d.

Table 6. *Analytical characteristics of udder-tissue fat and milk fat in* [1-^{14}C]iso*valeric acid experiment*

n.d., not determined.

	Udder tissue	Milk
	(g./100 g. of fat)	
Glyceride	89·0	88·6
Phospholipid	7·0	11·2
Total cholesterol	3·9	0·2
Distribution of fatty acids in glycerides as molar %		
Fatty acid		
Butyric	7·4±0·4	9·2±0·5
Caproic	2·3±0·2	3·3±0·2
Caprylic	1·5±0·1	1·00±0·05
Capric	5·6±0·3	1·30±0·06
Lauric	3·7±0·2	0·60±0·03
Myristic	7·5±0·4	
Palmitoleic	1·9±0·1	12·5±0·6
Linoleic	3·1±0·2	
Palmitic	28±1	19±1
Stearic	11·4±0·6	9·9±0·5
C_{14} unsaturated	0·50±0·03	n.d.
Oleic	27±1	38±2
Highly unsaturated acids	0·40±0·02	4·8±0·3

Table 7. *Recovery of* ^{14}C *in even-numbered fatty acid fractions in the experiment with* [1-^{14}C]*iso*-*valerate*

^{14}C recovered is expressed as percentage of total fat activities. —, no measurable activity.

Fatty acid	Udder tissue (%)	Milk (%)
Butyric	3·0±0·2	—
Caproic	1·9±0·1	—
Caprylic	3·2±0·3	6±1
Capric	26±2	12±1
Lauric	18±1	5±1
Myristic	23±2	—
Palmitic	24±2	77±8
Stearic	0·30±0·07	—
Palmitoleic	0·050±0·006	—
Oleic	1·0±0·1	—

Table 8. *Specific activities of the long-chain acids from udder tissue*

*iso*Valeric acid was used as precursor.

Acid	Counts/min./mg.
Lauric	491
Myristic	519
Branched pentadecanoic	598
n-Pentadecanoic	95
Palmitic	104
Branched heptadecanoic	110
n-Heptadecanoic	52
Stearic	5·6

Table 8). Approximately 20 % of the added ^{14}C was recovered in the various triglyceride fractions but little or no activity could be found in the isolated cholesterol.

The glycerides isolated from udder tissue were contaminated with cholesterol esters but determinations of free and total cholesterol showed that cholesterol esters were a minor component only.

Only a trace of activity could be detected in lactose.

Krebs-cycle acids. The specific activities of the isolated citric acid, succinic acid and malic acid were very high (Table 5), approximately 1·5 % of the added ^{14}C being present in citric acid. Unlike the fatty acid samples, the citric acid samples isolated from both udder tissue and milk had very similar specific activities.

DISCUSSION

The results reported demonstrate that although leucine is rapidly incorporated as such into milk proteins, especially casein, it acts neither as a precursor of fatty acids nor as an energy-yielding substrate.

*iso*Valeric acid, however, is readily metabolized and acts as a precursor of both fatty acids and certain of the non-essential amino acids. Its catabolic rate is about five times as great as that of leucine.

The specific activities of the fatty acids from the udder tissue were about 100 times as great as those of the acids found in milk. A similar effect was attributed by Cowie *et al.* (1951) to dilution of the secreted milk by residual milk present in the tissue at the beginning of the experiment. However, in the experiments reported here, citric acid from milk had a higher activity than that from udder tissue and, in an earlier experiment (Lauryssens, Peeters, Coussens & De Loose, 1957), lactose samples isolated from milk and udder tissue did not have the widely different specific activities found frequently for fatty acids from the two sources. These results cannot be explained by any dilution effect but must represent different rates of secretion of the milk constituents.

The labelling of the even-numbered fatty acids found in the udder as well as that of the Krebs-cycle acids can only be due to breakdown of the carboxyl-labelled *iso*valeric acid between C-2 and C-3 to give a reactive C_2 unit, presumably acetyl-coenzyme A. This agrees with the scheme of *iso*-valerate catabolism proposed by Greenberg (1954). The relative labelling of the different fatty acids is similar to that obtained from acetate (Popják, French, Hunter & Martin, 1951) in the living goat and from butyrate (Lauryssens, Verbeke & Peeters, 1957) in the perfused udder.

This breakdown of the *iso*valerate must be extremely rapid for, despite attempts to slow degradation reactions by continuous addition of acetate, no selective incorporation of *iso*valerate into the branched-chain acids could be demonstrated. Indeed the radioactivity of these branched-chain acids can almost wholly be accounted for by the incorporation of acetate alone into the molecule. The synthesis of similar branched-chain acids such as β-hydroxy-β-methylglutarate, β-hydroxy*iso*valerate and ββ-dimethylacrylate from precursors such as acetate has already been demonstrated (see Rudney, 1957). Clearly then, the presence of branched-chain saturated acids in milk cannot be due to chain lengthening of these branched-chain precursors known to be present in blood but is caused by net synthesis within the mammary gland from a substance such as acetate.

The high specific activity of the casein amino acids, glutamic acid and aspartic acid, and the lower activity of glycine, serine, alanine and lactose, can be explained by the active C_2 fragment from *iso*valerate entering the Krebs cycle (see Terner, 1951) and being further converted into oxaloacetate and pyruvate. Similar relative labelling of these compounds was found in perfusion experiments with [^{14}C]acetate and [^{14}C]butyrate (Verbeke, Aqvist & Peeters, 1957; Lauryssens, Verbeke & Peeters, 1957). The Krebs cycle in the mammary

gland therefore acts not only as an energy producer but also as a provider of the non-essential amino acids of milk protein.

SUMMARY

1. Two separated halves of lactating cow udders were perfused with blood containing respectively carboxyl-labelled leucine (0·95 mc of DL-[1-^{14}C]-leucine, the 'leucine half') and carboxyl-labelled *iso*valerate (0·5 mc of [1-^{14}C]*iso*valerate, the '*iso*-valerate half') for 2 hr. Both halves had inactive acetate added continuously throughout the perfusion.

2. The total quantity of ^{14}C recovered as carbon dioxide was 1 % in the 'leucine half' and 6 % in the '*iso*valerate half'.

3. [1-^{14}C]Leucine was incorporated to a large extent into milk protein.

4. In the '*iso*valerate half', ^{14}C was incorporated into fatty acids isolated from the milk and udder glycerides. The specific activity of the lower fatty acids increased with increasing chain length, to reach a maximum at C_{10}, and fell progressively with further increase in chain length. No specific incorporation of ^{14}C into branched-chain fatty acids could be demonstrated. Of the added ^{14}C, 20 % was found in the fat.

5. In the '*iso*valerate half', casein was the most active of the milk constituents isolated. Of the ^{14}C activity in this fraction, 98 % was due to glutamic acid and aspartic acid. The isolated lactose was slightly radioactive.

6. These results are consistent with the assumption that [1-^{14}C]*iso*valeric acid is broken down between C_2 and C_3, yielding a $^{14}CO_2H$-labelled C_2 component with high acetylating capacity. This component is metabolized by way of the Krebs cycle as shown by the isolation of very highly labelled citric acid, malic acid and succinic acid from the udder tissue.

7. In all the perfusion experiments carried out in presence of ^{14}C-labelled precursors, the specific activities of udder-fat fractions were higher than those of the corresponding milk fractions by a factor varying between 10 and 100. The difference in specific activity of citric acid in udder and in milk is relatively very small.

8. The significance of these findings in relation to milk secretion is discussed.

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REFERENCES

Block, R. F., Durrum, E. L. & Zweig, G. (1955). *A Manual of Paper Chromatography and Paper Electrophoresis*, p. 56. New York: Academic Press Inc.

Busch, H., Hurlbert, R. H. & Potter, V. R. (1952). *J. biol. Chem.* **196**, 717.

Cocking, E. C. & Yemm, E. W. (1954). *Biochem. J.* **58**, xii.

Conway, E. J. (1950). *Microdiffusion and Volumetric Error*, p. 142. London: Crosby, Lockwood and Son.

Cowie, A. T., Duncombe, W. G., Folley, S. J., French, T. H., Glascock, R. F., Massart, L., Peeters, G. J. & Popják, G. (1951). *Biochem. J.* **49**, 610.

El-Shazly, K. (1952*a*). *Biochem. J.* **51**, 640.

El-Shazly, K. (1952*b*). *Biochem. J.* **51**, 647.

Greenberg, D. M. (1954). *Chemical Pathways of Metabolism*, vol. 2, p. 66. New York: Academic Press Inc.

Hirs, C. H. W., Moore, S. & Stein, W. H. (1954). *J. Amer. chem. Soc.* **76**, 6063.

Hotis, R. P. & Miller, W. T. (1936). *Tech. Bull. U.S. Dep. Agric.*, no. 400.

Howard, G. A. & Martin, A. J. P. (1950). *Biochem. J.* **46**, 532.

Isherwood, F. A. (1946). *Biochem. J.* **40**, 688.

James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.

James, A. T. & Martin, A. J. P. (1956). *Biochem. J.* **63**, 144.

James, A. T., Peeters, G. & Lauryssens, M. (1956). *Biochem. J.* **64**, 726.

Kalbe, H. (1954). *Hoppe-Seyl. Z.* **297**, 19.

Kenny, A. P. (1952). *Biochem. J.* **52**, 611.

Lambert, M. & Neish, A. C. (1950). *Canad. J. Res.* **28**, 83.

Lauryssens, M., Peeters, G., Coussens, R. & De Loose, R. (1957). *Arch. int. Pharmacodyn.* **109**, 203.

Lauryssens, M., Verbeke, R. & Peeters, G. (1957). *Comm. int. Conf. Radioisotopes Sci. Res. Paris.*

Lindenbaum, A., Schubert, J. & Armstrong, W. D. (1948). *Analyt. Chem.* **20**, 1120.

Martin, A. J. P. & James, A. T. (1956). *Biochem. J.* **63**, 138.

Mendel, B. & Goldscheider, I. (1925). *Biochem. Z.* **164**, 163.

Moore, S. & Stein, W. H. (1949). *J. biol. Chem.* **178**, 53.

Natelson, S., Pincus, J. & Lugovoy, J. (1948). *J. biol. Chem.* **175**, 745.

Peeters, G. & Massart, L. (1952). *Arch. int. Pharmacodyn.* **91**, 388.

Popják, G. (1950). *Biochem. J.* **46**, 560.

Popják, G., French, T. H. & Folley, S. J. (1951). *Biochem. J.* **48**, 411.

Popják, G., French, T. H., Hunter, G. D. & Martin, A. J. P. (1951). *Biochem. J.* **48**, 612.

Popják, G., Glascock, R. F. & Folley, S. J. (1952). *Biochem. J.* **52**, 472.

Rudney, H. (1957). *J. biol. Chem.* **227**, 363.

Shorland, F. B. (1956). *Annu. Rev. Biochem.* **25**, 101.

Terner, C. (1951). *Biochem. J.* **50**, 145.

Trappe, W. (1938). *Biochem. Z.* **296**, 180.

Verbeke, R., Aqvist, S. & Peeters, G. (1957). *Arch. int. Physiol. Biochim.* **65**, 433.

Isolation of Lombricine and its Enzymic Phosphorylation

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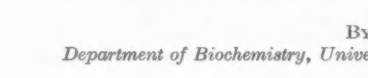
Van Thoai & Robin (1954) isolated a new mono-substituted guanidine diester from *Lumbricus terrestris* L., named lombricine, which played the role of phosphagen in the worms during muscular contraction. According to them the compound was guanidinoethyl seryl phosphate (I).



(I)

This paper deals with a modified method for the isolation of lombricine in improved yield and a study of some of its properties and its transphosphorylation under various conditions.

EXPERIMENTAL

Materials

Worms were collected locally when weather conditions favoured, otherwise they were obtained from L. Haig and Co., Beam Brook, Newdigate, Surrey, and identified as *Lumbricus terrestris* L.

Ion-exchange resins. $C_{50}D$ was a gift from Phillips and Pain-Vermorel, 31, Rue de la Vanne, Montrouge, Seine, France. It is a cross-linked divinylbenzene–polystyrene resin and a fairly strongly acidic cation-exchanger with SO_3H^- as the functional group. As the resin is supplied in the sodium form it was converted into its hydrogen form by soaking it in about twice its volume of HCl (10%, v/v) for 2–3 hr. with occasional stirring. The material was then transferred to a column 45 cm. in length and 2·5 cm. in diameter. Excess of acid was drained off and water passed up the tube to remove all air bubbles and the unduly fine particles of resin. When the washings leaving the column became clear, it was washed free from HCl by a downward flow of water to pH 5. In the text this column is referred to as the 'cation column $C_{50}D$'.

$A_{300}D$ was also given by Phillips and Pain-Vermorel. It is a divinylbenzene–polystyrene resin with amino and hydroxyamino groups as the functional groups and is a moderately basic anion-exchanger. The resin being supplied in the chloride form was used after soaking it in water (2 bed-volumes) for 2–3 hr. and back-washing in a column of 45 cm. in length and 2·5 cm. in diameter as described above. This column is referred to in the text as the '$A_{300}D$ anion column'.

Methods

Chromatography. Paper partition chromatography of Consden, Gordon & Martin (1944) was used. Both the one-dimensional ascending method and the two-dimensional technique of Datta, Dent & Harris (1950) were employed. Small volumes (20–30 μl.) of aqueous solutions of pure amino acids and worm extracts at various stages of isolation of lombricine were dried on Whatman no. 1 filter-paper sheets of suitable size (30 cm. × 20 cm.) in a stream of warm air (40–50°) from a hair-drier. Additions were made, 2–3 μl. at a time, drying between each addition and restricting the spot size to about 0·5 cm. in diameter.

One-dimensional chromatography. The chromatograms were developed at room temperature (22°) for about 6–8 hr. and the solvent was allowed to ascend a distance of about 23–25 cm. The operation was conducted in an air-tight glass aquarium tank fitted with a lid. The atmosphere of the tank was kept saturated with the vapours of the solvent.

Two-dimensional ascending chromatograms. Sheets (20 cm. × 20 cm.) were developed first with 80% (w/v) aq. phenol for 7–8 hr. in an atmosphere of NH_3 provided by the presence of a beaker of aq. NH_3 (sp.gr. 0·88). The second development was with 90% (v/v) aq. methyl Cellosolve (2-methoxyethanol; Bender, 1951) for 4–5 hr. as irrigating solvents.

Detection of spots and their identification. After the complete evaporation of the solvents from the chromatograms, the paper sheets were uniformly sprayed with a 0·5% (w/v) solution of ninhydrin in butanol. Spots, usually shades of purple, appeared after heating the papers for 5 min. at 110°. The amino acid spots were identified by comparing them with the two-dimensional array of spots obtained by preparing a chromatogram of known pure amino acids both singly and as mixtures under the same conditions. This chromatogram, referred to as a 'map of spots' by Dent (1948) for the particular pair of solvents used, was kindly supplied by Dr D. S. P. Patterson.

The spots on the one-dimensional chromatograms were identified by running spots of known pure substances under identical conditions on the same sheet of paper as markers along with a mixture of the known and the unknown amino acids.

For the detection of guanidine derivatives the chromatograms were first sprayed with a mixture of solutions containing 0·2 ml. of NaOH (40%, w/v), 0·2 ml. of ethanolic solution of α-naphthol (0·1%, w/v) and 0·2 ml. of urea solution (40%, w/v) in 10 ml. of water. The mixture was freshly prepared at the time of spraying. After the papers had been dried at room temperature they were sprayed with a solution of NaOBr obtained by diluting (1:5, v/v) a stock solution of 0·9% (v/v) bromine in 10% (w/v) NaOH.

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Appearance of pink spots indicated the presence of guanidine derivatives.

Buffers. Phthalate and borate buffers were prepared from 0·2 M-potassium hydrogen phthalate and 0·2 M-boric acid in 0·2 M-KCl respectively by adjusting the pH with 0·2 N-NaOH (Hawk, Oser & Summerson, 1954). Glycine (pH 9) was prepared by titrating a molar solution of glycine with N-NaOH. 2-Amino-2-hydroxymethylpropane-1:3-diol (tris, pH 7) was made from 0·25 M-solution by addition of 0·1 N-HCl (Gomori, 1955).

Isolation of lombricine from worms. Live worms (1 kg.) were freed from mud by washing them in cold water and were then homogenized with 1 l. of ice-cold (distilled) water in a Waring Blendor for 5 min. The homogenate was adjusted to pH 4 by adding conc. H_2SO_4 dropwise and boiled vigorously for 5 min. After cooling and centrifuging, the clear supernatant was removed and stored. The residue was washed with boiling water (200 ml.) and centrifuged and the supernatant was added to that first collected. This washing procedure was repeated once. To the combined supernatants was then added excess of basic lead acetate solution (20%, w/v; 450 ml.) till there was no further precipitation. The mixture was well stirred and left overnight at room temperature. The clear supernatant was sucked off and the precipitate (lead proteinate) centrifuged down and washed three times with dilute basic lead acetate solution (5%, w/v; 100 ml. portions). The precipitate was discarded and the washings were collected and added to the clear supernatant. A current of H_2S was then passed through the worm extract for 5–6 hr. The mixture was centrifuged and the supernatant retained; the precipitated lead sulphide was washed thoroughly (4–5 times) with saturated aq. H_2S to extract the guanidine bases adsorbed. The combined lead-free extracts were concentrated to about 200 ml. under reduced pressure and filtered. The extract (pH 8·5) was brought to pH 4 by the dropwise addition of conc. H_2SO_4 and was passed through the cation column C_{50}D at the rate of 8–10 drops (approx. 0·8–1·0 ml.)/min. Acidic amino acids passed in the effluent, whereas the basic and the neutral ones were adsorbed on the column. After the column had been washed with water until the pH of the effluent rose to 5 it was eluted with aq. 0·15 N-NH_3 till the eluate became negative to the Sakaguchi test. The eluate contained the base lombricine, some neutral acids like alanine, serine and leucine, etc., and traces of the stronger bases arginine and lysine.

Ammonia was then removed by concentrating the eluate *in vacuo* to 100 ml. This concentrate (pH 8·3) was passed through the anion column A_{300}D which allowed lombricine along with traces of arginine to pass into the effluent, whereas all the other remaining amino acids, e.g. lysine, serine, alanine, tyrosine and phenylalanine, were adsorbed on the column. The column was washed with water until the effluent became negative to the Sakaguchi test and the combined effluents were evaporated to dryness *in vacuo*. The residue, consisting mainly of lombricine, was dissolved, in the smallest possible quantity (50–60 ml.) of hot aqueous ethanol (50%, v/v) and filtered. Absolute ethanol was then added in small quantities (approx. 150 ml.) till a turbidity was produced and the solution was left at 0° overnight. The white crystalline base (0·85 g.) was filtered off, washed with ethanol 2–3 times and finally with ether and dried. The solid material was recrystallized several times from aqueous ethanol and finally from water. Yield 0·60 g.; m.p. 228° [cf. yield 0·06 g.; m.p. 223–224° (Van Thoai & Robin, 1954)].

Isolation of lombricine from lombricine flavianate. As a modification to the method described above, lombricine was isolated by converting the base present in the concentrated eluate from the C_{50}D column into lombricine flavianate by treating it with flavianic acid (2:4-dinitro-1-naphthol-7-sulphonic acid; Stein, Moore & Bergmann, 1944) and by passing a 2% (w/v) solution of the crystallized flavianate through the anion column A_{300}D to remove the flavianic acid. The Sakaguchi-positive effluent was collected, concentrated *in vacuo* and purified by repeated crystallization from water. Yield 0·317 g./kg. of worms; m.p. 225–226°.

The material obtained by both the methods described ran as a single spot on the paper chromatogram in all the solvents tested (2–9, Table 1). The spot gave positive reactions with ninhydrin and Sakaguchi reagents. Spraying with the molybdate reagent of Hanes & Isherwood (1949) showed the presence of phosphate.

The melting point was unchanged on mixing with an authentic specimen of lombricine (kindly supplied by Mme Y. Robin of Collège de France, Paris) (Found: C, 26·4; H, 5·6; N, 20·67; P, 11·7. $C_6H_{15}O_6N_4P$ requires C, 26·6; H, 5·5; N, 20·85; P, 11·4%).

The flavianate of lombricine, prepared in the usual manner, crystallized from water in small shining orange needles and melted above 250° with decomposition (Found: N, 14·3; P, 5·4. $C_{16}H_{19}O_{13}N_{16}SP$ requires N, 14·8; P, 5·5%).

Attempts to prepare the picrate and the benzoyl derivative of lombricine proved unsuccessful.

Table 1. *R_F of lombricine developed with different solvent systems*

Solvent	R_F
1	No movement
2	0·06
3	0·15
4	0·11
5	0·05
6	0·05
7	0·17
8	0·48
9	0·06

The proportions of the components of the solvent systems are by volumes. 1, butan-1-ol–water–acetic acid (73:17:10); 2, pyridine–*iso*amyl alcohol–water–acetic acid (80:40:40:10); 3, pyridine–*iso*amyl alcohol–water (80:40:70); 4, pyridine–*iso*amyl alcohol–water–20% (v/v) aq. NH_3, sp.gr. 0·88 (80:40:40:10); 5, phenol saturated with water; 6, propan-1-ol–water–20% (v/v) aq. NH_3, sp.gr. 0·88 (73:7:20); 7, methanol–water–acetic acid (80:20:10); 8, phenol (80%, w/v)–NH_3 (see text); 9, aqueous methyl Cellosolve (90%).

Enzymic experiments

Acetone-dried powder of worms. This was prepared by the method of Green, Needham & Dewan (1937). Worms (50 g.) were weighed in an ice-cold beaker and thoroughly ground up in an ice-cold mortar with 100 ml. of water at 0° and allowed to stand for 10 min. Ice-cold acetone (300 ml.) was then stirred into the worm extract for 2–3 min. and the

suspension filtered through a Büchner funnel, with gradually increasing suction. When the powder became fairly dry it was tipped back into the mortar, re-extracted with ice-cold acetone (125 ml.), thoroughly mixed and filtered off in the same filter paper used previously. The brick-red powder obtained was washed with a little cold acetone (20 ml.) and sucked almost dry, care being taken to avoid undue exposure of the powder to the atmosphere. It was then dried *in vacuo* over liquid paraffin and stored at 0°.

Preparation of the enzyme from the acetone-dried powder. A sample of the powder (5 g.) was mixed with 15 ml. of water in a mortar to obtain a homogeneous paste. This was then dialysed at 0° for 15 hr. against water in a cellophan sac. The contents of the sac were centrifuged to remove the insoluble matter and the clear supernatant was used for enzymic experiments.

Preparation of the worm homogenate. Clean live worms (300 g.) were cooled to −5° in a beaker for about 30 min. and homogenized in a Waring Blendor with water (150 ml.) at 0°. The very frothy homogenate was squeezed through a cheese cloth, the pasty residue being discarded. The strained, frothy, viscous liquid (about 120 ml.) obtained was dialysed at 0° for 15 hr. in a number of cellophan sacs against 15 l. of water in a glass tank. The large amount of insoluble material was centrifuged off and discarded. After the clear, pink supernatants (150 ml.) had been mixed, they were stored in 10 ml. portions at −20°.

Transphosphorylation of lombricine. Six tubes with different incubation systems were set up (Table 2) by adding, in order, 0·1 ml. of 0·01 M-$MgSO_4$, 0·75 ml. of 0·1 M-lombricine, 0·75 ml. of 0·1 M-arginine, 0·15 ml. of 0·1 M-adenosine triphosphate (ATP), 0·30 ml. of buffer solution and 1 ml. of the enzyme solution. The final volume of all the tubes was made up to 3 ml. with water and the tubes were incubated for 15 min. at 37°, after which they were cooled in an NaCl–ice mixture. When cool, 1 ml. of trichloroacetic acid (32%, w/v) was added to each tube and the contents centrifuged at 0°. The supernatant was decanted from the precipitated protein and immediately neutralized with NaOH (40%, w/v), phenolphthalein being used as indicator. Then 2 ml. of aq. 10% (w/v) $CaCl_2$ at 0°, saturated with $Ca(OH)_2$, was added and the tubes were allowed to stand at 0° for 15 min. The precipitate of inorganic phosphates was spun off and discarded, the supernatant filtered and the volume was made up to 10 ml. with water. To 5 ml. of this, 1 ml. of trichloroacetic acid (10%, w/v) was added and the phosphorylated compound was hydrolysed for 1 min. in a boiling-water bath. After cooling, 2·5 ml. of ammonium molybdate [2·5% (w/v) in 5 N-H_2SO_4] was added to each tube, followed by Fiske-Subbarow reducing agent (1 ml.). The volume was made up to 10 ml. and the blue colour developed after 15 min. was compared against a standard solution containing 0·1 mg. of P/ml. in a Gallenkamp colorimeter no. 3615 with 608 mμ (red) filter.

RESULTS

Enzymic phosphorylation of lombricine was attempted with acetone-dried powder of the worms from pH 4·6 to 10 (phthalate buffers, 4·6–6; tris, 7; borate buffers, 8–10 and glycine buffer 9), dialysing the enzyme against water for different lengths of time (15 and 48 hr.) and employing different makes of ATP (L. Light and Co., and Sigma Chemical Co. Ltd.).

Only in glycine–HCl buffer (pH 9·0) was there an appreciable transphosphorylation. The amount of phosphagen P formed under these conditions was roughly one-half that obtained with a worm homogenate under the same conditions (Table 2). Increasing the dialysis time from 15 to 48 hr. decreased the amount of phosphagen P formed by the acetone-dried powder preparation by 60%, while the phosphagen P formed by the worm homogenate fell by 40%. Omission of Mg^{2+} ions from the reaction mixture caused a decreased formation of phosphagen P. This effect was greatest after prolonged dialysis (48 hr.). The reduction was 34% with the acetone-dried powder and 38% with the homogenate, whereas after 15 hr. dialysis the reduction was only 20 and 10% respectively.

By using the worm homogenate in glycine–HCl buffer (pH 9), guanidinoethanol phosphate and taurocyamine were successfully phosphorylated, the phosphagen P formed in 5 ml. each of the incubated mixtures being 19·60 and 18·40 μg. respectively. However, when arginine, creatine, guanidine, guanidinoethanol and glycocyamine were similarly treated they did not accept the phosphate groups at the expense of ATP.

Table 2. *Transphosphorylation of lombricine with acetone-dried powder and worm homogenate with* M-*glycine buffer at* pH 9

The complete incubation system comprised: 0·10 ml. of 0·01 M-$MgSO_4$, 0·75 ml. of 0·1 M-lombricine, 0·15 ml. of 0·1 M-ATP, 0·3 ml. of buffer and 1 ml. of enzyme solution. The values are presented after subtracting the apparent phosphagen P formed in the absence of lombricine. No appreciable phosphagen P was formed when enzyme or ATP was omitted or when arginine was substituted for lombricine.

Enzyme source	Time of dialysis (hr.)	μg. of phosphagen P formed in incubation systems	
		With Mg^{2+}	Without Mg^{2+}
Acetone-dried powder	15	10·20	8·20
Acetone-dried powder	48	4·20	2·80
Worm homogenate	15	26·50	22·40
Worm homogenate	48	15·92	9·80

DISCUSSION

The progressive loss of activity of the enzyme preparations during dialysis suggested that some cofactor was being removed from the enzyme by the treatment. This was tested by adding a concentrate of the diffusate to the enzyme preparations, but no reactivation of the enzyme preparations was obtained. This suggests that, if loss of activity of the enzymes is due to a loss of a cofactor, then this cofactor must be labile under the conditions of dialysis and subsequent concentration of the aqueous diffusate.

The bases tested as acceptors in the transphosphorylation reaction may be divided into two classes: those containing acidic groups such as phosphate or sulphate and those without acidic groups. It may be of significance that only the acid-containing bases (lombricine, guanidinoethanol phosphate and taurocyamine) were phosphorylated.

SUMMARY

1. Lombricine has been isolated in good yield from *Lumbricus terrestris* L. with ion-exchange resins. Lombricine flavianate has been prepared.

2. Transphosphorylation from adenosine triphosphate to lombricine has been achieved with dialysed enzyme preparations from worm homogenates or acetone-dried powders. The addition of Mg^{2+} ions was necessary for maximum enzyme activity.

3. Of the bases other than lombricine tested (guanidinoethanol phosphate, taurocyamine, arginine, creatine, guanidine, guanidinoethanol and glycocyamine) as phosphate acceptors, only guanidinoethanol phosphate and taurocyamine were active.

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REFERENCES

Bender, A. E. (1951). *Biochem. J.* **48**, xv.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Datta, S. P., Dent, C. E. & Harris, H. (1950). *Science*, **112**, 621.
Dent, C. E. (1948). *Biochem. J.* **43**, 168.
Gomori, G. (1955). *Methods in Enzymology*, vol. 1, p. 138. Ed. by Kaplan, N. O. & Colowick, S. P. New York: Academic Press Inc.
Green, D. E., Needham, D. M. & Dewan, J. G. (1937). *Biochem. J.* **31**, 2327.
Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
Hawk, P. B., Oser, B. L. & Summerson, W. H. (1954). *Practical Physiological Chemistry*, 13th ed. London: J. and A. Churchill Ltd.
Stein, W. H., Moore, S. & Bergmann, M. (1944). *J. biol. Chem.* **154**, 191.
Van Thoai, N. & Robin, Y. (1954). *Biochim. biophys. Acta*, **14**, 76.

Pepsin-Catalysed Transpeptidation of the Amino-Transfer Type

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Some *N*-acyldipeptides containing tyrosine or phenylalanine or both are known to be hydrolysed by pepsin (Fruton & Bergmann, 1939; Harington & Pitt-Rivers, 1944; Dekker, Taylor & Fruton, 1949; Casey & Laidler, 1950; Baker, 1951). Direct cleavage of the peptide bond, producing an acylamino acid and a free amino acid, was the only reaction assumed to take place. Analysis of the products formed by peptic digestion of substrates of the above type, containing a *C*-terminal tyrosine, showed that both transpeptidation and hydrolysis occur. It could furthermore be demonstrated that the transpeptidation involves an amino transfer, where the amino moiety of the tyrosine peptides used as substrates is transferred to an acceptor with a free carboxyl group (Hanes, Hird & Isherwood, 1950).

EXPERIMENTAL

Materials

Twice-crystallized pepsin from Mann Research Laboratories Inc., New York, was used throughout.

The following substances were prepared according to the literature: *N*-acetyl-L-tyrosine (du Vigneaud & Meyer, 1932); *N*-benzyloxycarbonyl-L-tyrosyl-L-tyrosine and *N*-benzyloxycarbonyl-L-glutamyl-L-tyrosine (Bergmann, Zervas, Salzmann & Schleich, 1934); *N*-benzyloxycarbonyl-L-tyrosyl-L-tyrosyl-L-tyrosine (*N*-benzyloxycarbonyltri-L-tyrosine, Barkdoll & Ross, 1944); *N*-benzyloxycarbonyl-L-glutamyl-L-tyrosine amide (Fruton & Bergmann, 1939); *N*-benzyloxycarbonyl-L-glutamic acid (Bergmann & Zervas, 1932); L-tyrosyl-L-tyrosine (Barkdoll & Ross, 1944); *N*-benzyloxycarbonyl-L-tyrosine (Bergmann & Zervas, 1932); L-tyrosine amide (Blau & Waley, 1954). *NO*-Dibenzyloxycarbonyl-L-tyrosine was prepared according to Katchalski & Sela (1953); good yields were obtained when care was taken to maintain pH 9–10 during the coupling. The amount of 4N-NaOH (40 ml.) given by Katchalski & Sela (1953) is in error. Only about 15 ml. was necessary for the coupling of 11·5 g. of tyrosine.

NO-*Dibenzyloxycarbonyl*-L-*tyrosyl*-L-*tyrosine ethyl ester* (I). This compound was prepared from *NO*-dibenzyloxycarbonyl-L-tyrosine (4·5 g.), *iso*butyl chlorocarbonate (1·37 g.), triethylamine (1 g.) and L-tyrosine ethyl ester (2·1 g.) in ethyl acetate, by the procedure of Vaughan & Osato (1952). Yield was 5 g. Recrystallization from ethanol gave 4·2 g. of *NO*-dibenzyloxycarbonyl-L-tyrosyl-L-tyrosine ethyl ester, m.p. 178–179° (Found: C, 67·5; H, 5·7; N, 4·3. $C_{36}H_{36}N_2O_8$ requires C, 67·5; H, 5·7; N, 4·4%).

L-*Tyrosyl*-L-*tyrosine ethyl ester hydrobromide* (II). Compound (I) (2·5 g.) was treated with anhydrous HBr (30% in acetic acid) at room temperature (Ben-Ishai & Berger, 1952) in the presence of phenol (1 g.). On addition of anhydrous ether, after 20 min. an oil precipitated which was repeatedly washed with ether. Reprecipitation with ether from ethanol solution gave 1·4 g. of (II), m.p. 218–220° (Found: C, 52·7; H, 5·5; N, 5·9; Br, 17·7. $C_{20}H_{24}N_2O_5$,HBr requires C, 53·0; H, 5·6; N, 6·2; Br, 17·6%).

NOO′-*Triacetyl*-L-*tyrosyl*-L-*tyrosine ethyl ester* (III). Compound (II) (3 g.) was dissolved in anhydrous pyridine, and acetic anhydride (10 ml.) was added. After 24 hr. the reaction mixture was poured into ice water and acidified with conc. HCl. The triacetyl-L-tyrosyl-L-tyrosine ethyl ester was extracted from the above mixture with ethyl acetate, dried over anhydrous Na_2SO_4 and reprecipitated with light petroleum. Recrystallization from methanol gave 2·6 g. of (III), m.p. 99° (Found: C, 63·2; H, 5·9; N, 5·9. $C_{26}H_{30}N_2O_8$ requires C, 62·6; H, 6·1; N, 5·6%).

N-*Acetyl*-L-*tyrosyl*-L-*tyrosine*. Compound (III) (2·5 g.) was dissolved in ethanol, and N-NaOH (25 ml.) was added. After 30 min. the solution was acidified with conc. HCl to pH 5, concentrated *in vacuo* and the residue dissolved in ethyl acetate. On precipitation with light petroleum an oil was obtained, which after crystallization from methanol gave 0·8 g. of *N*-acetyl-L-tyrosyl-L-tyrosine. $[\alpha]_D^{25}+19°$ in ethanol (*c*, 1·5), m.p. 240° [Baker (1951) gives $[\alpha]_D^{26}+19°$ in ethanol, m.p. 241°] (Found: C, 62·0; H, 5·7; N, 7·2; $CH_3{\cdot}CO$, 12·0. $C_{20}H_{22}N_2O_6$ requires C, 62·2; H, 5·7; N, 7·2; $CH_3{\cdot}CO$, 11·2%).

N-p-*Nitrobenzoyl*-L-*tyrosine ethyl ester* (IV). This compound was prepared by coupling *p*-nitrobenzoyl chloride (9·2 g.) with L-tyrosine ethyl ester hydrochloride (12·2 g.) in 500 ml. of chloroform, in the presence of 100 ml. of aqueous N-Na_2CO_3. Yield was 13 g., m.p. 139–140° (Found: C, 60·2; H, 4·9; N, 7·8; C_2H_5O, 13·0. $C_{18}H_{18}N_2O_6$ requires C, 60·3; H, 5·1; N, 7·8; C_2H_5O, 12·6%).

N-p-*Nitrobenzoyl*-L-*tyrosine hydrazide* (V). Compound (IV) (7 g.) was dissolved in anhydrous ethanol (50 ml.) and refluxed for 2 hr. with hydrazine hydrate (2 g.). *N*-*p*-Nitrobenzoyl-L-tyrosine hydrazide (5 g.) precipitated, m.p. 265° (Found: C, 55·8; H, 4·7; N, 16·3; equiv. wt. 355 by titration with perchloric acid in acetic acid and methyl violet as indicator. $C_{16}H_{16}N_4O_5$ requires C, 55·8; H, 4·7; N, 16·3%; equiv. wt. 344).

N-p-*Nitrobenzoyl*-L-*tyrosyl*-L-*tyrosine ethyl ester* (VI). A solution of (V) (4 g.) in acetic acid was mixed with 100 ml. of ethyl acetate, 20 ml. of 2N-HCl and 50 ml. of water. To the ice-cooled mixture $NaNO_2$ (900 mg.) in 2 ml. of cold water was added with shaking. The organic layer, containing *p*-nitrobenzoyltyrosine azide, was separated, washed with water and aqueous bicarbonate, and dried over anhydrous Na_2SO_4. The dried azide solution was mixed with an ice-cooled solution of tyrosine ethyl ester (3 g.) in ethyl acetate (20 ml.). After standing overnight, *p*-nitrobenzoyl-L-tyrosyl-L-tyrosine ethyl ester (1·6 g.) precipitated, m.p. 185–187° (Found: C, 62·0; H, 5·3; N, 8·1; C_2H_5O, 8·5. $C_{27}H_{27}N_3O_8$ requires C, 62·2; H, 5·2; N, 8·1, C_2H_5O, 8·6%).

N-p-*Nitrobenzoyl*-L-*tyrosyl*-L-*tyrosine* (VII). This compound was prepared by shaking (VI) with 3 equiv. of N-NaOH until dissolved (the solution became red). After 1 hr. *p*-nitrobenzoyl-L-tyrosyl-L-tyrosine was precipitated by acidification. It was extracted into ethyl acetate, the solution dried and the product was precipitated by light petroleum, m.p. 218–219° (Found: C, 58·8; H, 4·9; N, 8·2. $C_{25}H_{23}N_3O_8,H_2O$ requires C, 58·7; H, 4·9; N, 8·2%).

N-p-*Aminobenzoyl*-L-*tyrosyl*-L-*tyrosine*. This compound was obtained by catalytic reduction of (VII) in ethanol at room temperature and 2 atm. pressure in the presence of Pd (5% on charcoal). The reaction mixture was filtered, and concentrated *in vacuo*. On precipitation with water *N*-*p*-aminobenzoyl-L-tyrosyl-L-tyrosine, m.p. 246–249°, was obtained (Found: C, 62·3; H, 5·4; N, 8·7. $C_{25}H_{25}N_3O_6,H_2O$ requires C, 62·4; H, 5·6; N, 8·7%).

N-p-*Nitrobenzoyl*-L-*tyrosine* (VIII). This compound was prepared by alkaline hydrolysis of (IV), with 2 equiv. of 2N-NaOH, analogously to the preparation of (VII). On acidification *p*-nitrobenzoyl-L-tyrosine was precipitated, m.p. 176–177° (Found: C, 57·8; H, 4·2; N, 8·5. $C_{16}H_{14}N_2O_6$ requires C, 58·2; H, 4·3; N, 8·5%).

N-p-*Aminobenzoyl*-L-*tyrosine*. This compound was obtained by catalytic reduction of (VIII) in ethanol at room temperature and 1 atm. in the presence of Pd (5% on charcoal). The ethanol was evaporated *in vacuo* and the residue dried in a desiccator. When the crude product was treated with hot ethyl acetate it first dissolved and then crystallized out on further heating. *N*-*p*-Aminobenzoyl-L-tyrosine was recrystallized from aq. 85% (v/v) ethanol, m.p. 267° (Found: C, 63·5; H, 5·0; N, 9·5. $C_{16}H_{16}N_2O_6$ requires C, 64·0; H, 5·4; N, 9·3%).

N-*Benzyloxycarbonyl*-L-*phenylalanyl*-L-*tyrosine* (IX). This compound was obtained by alkaline (2 moles of 2N-NaOH) hydrolysis of *N*-benzyloxycarbonyl-L-phenylalanyl-L-tyrosine ethyl ester (Vaughan & Osato, 1952) in ethanol, m.p. 183–184° (Found: C, 67·4; H, 5·6; N, 6·1. $C_{26}H_{26}N_2O_6$ requires C, 67·5; H, 5·7; N, 6·1%).

L-*Phenylalanyl*-L-*tyrosine hydrobromide*. Compound (IX) was treated with anhydrous HBr (Ben-Ishai & Berger, 1952). The peptide was isolated as the hydrobromide by precipitation with anhydrous ether, followed by repeated

washing with ether (Found: Br, 19·2. $C_{18}H_{20}N_2O_4$,HBr requires Br, 19·5%).

N-Benzyloxycarbonyl-γ-benzyl-L-glutamyl-L-tyrosyl-L-tyrosine ethyl ester (X). This compound was prepared from *N*-benzyloxycarbonyl-γ-benzyl-L-glutamate (740 mg.) (Hanby, Waley & Watson, 1950), triethylamine (400 mg.), *iso*butyl chlorocarbonate (275 mg.) and tryosyltyrosine ethyl ester hydrobromide (900 mg.), by the procedure of Vaughan & Osato (1952). Yield was 1·2 g., m.p. 141–143° (Found: C, 65·9; H, 6·2; N, 5·7. $C_{50}H_{43}N_3O_{10}$ requires C, 66·2; H, 5·9; N, 5·8%).

N-Benzyloxycarbonyl-L-glutamyl-L-tyrosyl-L-tyrosine. Compound (X) was dissolved in a minimal amount of ethanol and a slight excess (4·5 moles) of 2N-NaOH was added. After half an hour the solution was diluted with water and acidified, whereupon benzyloxycarbonyl-L-glutamyl-L-tyrosyl-L-tyrosine was precipitated, m.p. 177–178° (Found: C, 61·2; H, 5·5; N, 7·0. $C_{31}H_{33}N_3O_{10}$ requires: C, 61·3; H, 5·5; N, 6·9%).

NO-Dibenzyloxycarbonyl-L-tyrosyl-L-tyrosyl-L-tyrosine ethyl ester (XI). Dibenzyloxycarbonyl-L-tyrosine (4·5 g.) (Katchalski & Sela, 1953) was treated with triethylamine (2 g.), *iso*butyl chlorocarbonate (1·37 g.) and L-tyrosyl-L-tyrosine ethyl ester hydrobromide (4·5 g.), by the procedure of Vaughan & Osato (1952). Dibenzyloxycarbonyltrityrosine ethyl ester thus obtained had m.p. 143–144°, yield 5·2 g. (Found: C, 67·2; H, 5·8; N, 5·1. $C_{45}H_{45}N_3O_{11}$ requires C, 67·2; H, 5·6; N, 5·2%).

Tri-L-tyrosine ethyl ester hydrobromide (XII). This compound was prepared from (XI) and anhydrous HBr, by the procedure of Ben-Ishai & Berger (1952); m.p. 167–170° after precipitation from ethanol by means of ether (Found: Br, 12·8. $C_{29}H_{33}N_3O_7$,HBr requires Br, 13·1%).

N-Benzyloxycarbonyl-γ-benzyl-L-glutamyltri-L-tyrosine ethyl ester (XIII). This compound was prepared by the mixed-anhydride method (Vaughan & Osato, 1952) from *N*-benzyloxycarbonyl-γ-benzyl-L-glutamate (3·5 g.) (Hanby *et al.* 1950), triethylamine (1 g.), *iso*butyl chlorocarbonate (1·37 g.) and trityrosine ethyl ester (4·3 g.). Yield was 60%, m.p. 167–169° (Found: C, 65·8; H, 5·8; N, 5·9. $C_{68}H_{52}N_4O_{12}$ requires C, 66·2; H, 5·9; N, 6·3%).

N-Benzyloxycarbonyl-L-glutamyltri-L-tyrosine. Compound (XIII) (0·5 g.) was shaken with 2·5 ml. of N-NaOH for half an hour. The solution was filtered and acidified with 2N-HCl. The *N*-benzyloxycarbonylglutamyltrityrosine which precipitated was dissolved in ethyl acetate and reprecipitated with light petroleum; m.p. 155–157°. Recrystallization from 85% (v/v) ethanol gave m.p. 179–180° (Found: C, 61·8; H, 5·8; N, 7·6. $C_{40}H_{42}N_4O_{12}$ requires C, 62·3; H, 5·5; N, 7·3%).

Methods

Buffers. In the pH range 3·7–5·0, acetic acid–sodium acetate buffers with the following composition were used: 0·85M-acetic acid, 0·1M-sodium acetate (pH 3·7); 0·28M-acetic acid, 0·1M-sodium acetate (pH 4·2); 0·15M-acetic acid, 0·1M-sodium acetate (pH 4·5); 0·09M-acetic acid, 0·1M-sodium acetate (pH 4·7); 0·05M-acetic acid, 0·1M-sodium acetate (pH 5·0).

In the pH range 1·7–3·5, HCl–sodium acetate buffers with the following composition were used: 0·22M-HCl, 0·20M-sodium acetate (pH 1·7); 0·21M-HCl, 0·20M-sodium acetate (pH 2·0); 0·204M-HCl, 0·20M-sodium acetate (pH 2·3); 0·199M-HCl, 0·20M-sodium acetate (pH 2·7); 0·194M-HCl, 0·20M-sodium acetate (pH 3·1); 0·18M-HCl, 0·20M-sodium acetate (pH 3·5).

The following phosphate buffers were used in the electrophoresis experiments: 0·05M-Na_2HPO_4, 0·05M-NaH_2PO_4 (pH 6·8); 0·10M-Na_2HPO_4 (pH 8·5).

Enzymic reaction. Reaction mixtures contained a total of 15–50 μmoles of substrate, and from 3 to 20 mg. of pepsin, per ml. The solvents were acetate buffers at pH 3·5–5·0, or HCl–sodium acetate buffers at pH 1·7–3·5. In the control experiments either the substrate or the enzyme was omitted. Because of the low solubility of most of the substrates in the pH range employed, they were dissolved in ethanol, or in the minimal amount of 0·2M-NaOH, before adjusting the pH with 0·2M-HCl and diluting the solution to the final volume with buffer. The final concentrations of ethanol did not exceed 10%. In some cases the substrates precipitated upon adjustment of the pH but redissolved during the course of incubation. The enzymic reactions were carried out at 37±1°.

Analysis of reaction products. For chromatographic analysis samples of 10–20 μl. of the incubation mixture were placed on Whatman no. 1 filter paper and developed with either butanol–acetic acid–water (25:6:25, by vol.) or propanol–water–conc. aq. NH_3 soln. ammonia (100:50:1, by vol.).

Paper electrophoresis was carried out in a Consden & Stanier (1952) apparatus with a potential gradient of 10 v/cm., with phosphate buffer, pH 8·5 for acetyltyrosyltyrosine and pH 6·8 for the other substrates.

Ninhydrin was used to locate the products containing free amino groups. Tyrosine and its derivatives were detected with diazotized sulphanilic acid in aq. 10% (w/v) Na_2CO_3 (Cramer, 1955), and *p*-aminobenzoyl derivatives with 2% (w/v) *p*-dimethylaminobenzaldehyde in 5% (w/v) HCl. This reagent produces a yellow with aromatic amines, which is strongly fluorescent in ultraviolet light (Dalgliesh, 1952).

As benzyloxycarbonyl-L-glutamic acid and benzyloxycarbonyl-L-phenylalanine could not be detected by these reagents, they were decarbobenzoxylated on the paper with HBr gas, and the free glutamic acid and phenylalanine formed were located with ninhydrin as follows: the paper was placed in a desiccator, which was evacuated and then filled with HBr gas, for 15–30 min. After evacuation to remove the HBr, the paper was washed with ether, dried in a stream of warm air (50–60°) and sprayed with ninhydrin containing pyridine. The blue spots obtained faded within 2 days. Benzyloxycarbonyl-L-glutamyl-L-tyrosine and benzyloxycarbonyl-L-phenylalanyl-L-tyrosine gave faint spots with ninhydrin under these conditions.

Abbreviations

The following abbreviations are used: Tyr for L-tyrosine and L-tyrosyl residues, Phe for L-phenylalanyl residues, Glu for L-glutamyl residues, Ac for acetyl, Z for benzyloxycarbonyl, PAB for *p*-aminobenzoyl and Am for amide groups.

RESULTS

N-Acetyl-L-tyrosyl-L-tyrosine (Ac.Tyr_2). When Ac.Tyr_2 was incubated with pepsin for varying time intervals (up to 48 hr.) at pH 2·0–4·7 and the mixture was analysed chromatographically with

butanol–acetic acid–water as developer, two ninhydrin-positive spots appeared, whose intensity increased with time. One spot had R_F 0·45, identical with that of a sample of tyrosine, and the other spot had R_F 0·68, identical with that of an authentic sample of Tyr_2. The eluted spot with R_F 0·68 yielded only tyrosine on acid hydrolysis. In experiments conducted at pH 2·0–3·5 (HCl–NaCl in the absence of sodium acetate) no formation of acetic acid could be detected either by the method of Hutchens & Kass (1949), or by chromatography according to Kennedy & Barker (1951). This excludes the possibility that Tyr_2 is formed by the hydrolysis of the *N*-acetyl bond of Ac.Tyr_2. (Pepsin did not hydrolyse *N*-acetyl-L-tyrosine in the pH range 2·0–4·7.)

The formation of Tyr_2 in the above experiments may be explained by the following sequence of reactions, involving transpeptidation as well as hydrolysis:

$$\text{Ac.Tyr}_2 + \text{Ac.Tyr}_2 \rightarrow \text{Ac.Tyr} + \text{Ac.Tyr}_3$$
$$\text{Ac.Tyr}_3 \rightarrow \text{Ac.Tyr} + \text{Tyr}_2$$
or $$\text{Ac.Tyr}_3 \rightarrow \text{Ac.Tyr}_2 + \text{Tyr}$$

N-p-*Aminobenzoyl*-L-*tyrosyl*-L-*tyrosine* (PAB.Tyr_2). This compound was chosen because of the ease with which *p*-aminobenzoic acid can be detected, even in traces, on paper chromatograms.

Chromatograms of 24 hr. incubation mixtures of PAB.Tyr_2 and pepsin at pH 1·7–4·7 developed with butanol–acetic acid–water, revealed, with ninhydrin, tyrosine (R_F 0·45) as well as tyrosyltyrosine (R_F 0·68). Parallel chromatograms with propanol–water–conc. aq. NH_3 soln. as developer revealed with *p*-dimethylaminobenzaldehyde two yellow spots with R_F 0·76 and 0·83, corresponding to those of the synthetic PAB.Tyr and PAB.Tyr_2 respectively. No trace of free *p*-aminobenzoic acid (R_F 0·51 in propanol–water–conc. aq. NH_3 soln.) was detected. Electrophoretic papers (pH 6·8) run for 2 hr. revealed with the same reagent two yellow spots, 5·3 cm. and 7·5 cm. from the origin towards the cathode, corresponding to those of the synthetic PAB.Tyr and PAB.Tyr_2 respectively. No traces of *p*-aminobenzoic acid, which moves 11 cm. from the origin towards the cathode under these conditions, was detected.

The absence of *p*-aminobenzoic acid in the peptic digest shows that the formation of Tyr_2 involved a transpeptidation reaction corresponding to that formulated above for Ac.Tyr_2, and did not result from the hydrolysis of the PAB.Tyr_2.

N-*Benzyloxycarbonyl*-L-*tyrosyl*-L-*tyrosine* (Z.Tyr_2). Chromatographic analysis of a 24 hr. peptic digest of Z.Tyr_2 (pH 4·7), with butanol–acetic acid–water as developer, revealed the formation of Tyr and Tyr_2. Since Z.Tyr is resistant to pepsin it may be assumed that the formation of Tyr_2 proceeds by a mechanism analogous to the one suggested to explain its formation from Ac.Tyr_2 and PAB.Tyr_2.

N-*Benzyloxycarbonyl*-L-*glutamyl*-L-*tyrosine* (Z.Glu.Tyr). Chromatographic analysis of the reaction mixture of pepsin and Z.Glu.Tyr at pH 4·5, with butanol–acetic acid–water as developer, revealed with ninhydrin the presence of Tyr (R_F 0·45) and Tyr_2 (R_F 0·68) in the digest. No spot corresponding to Glu.Tyr (Bergmann *et al.* 1934) (R_F 0·52) could be detected on the chromatogram.

The appearance in a proteolytic digest of a product with an amino acid sequence not present in the substrate is evidence for the occurrence of transpeptidation. Special care was therefore taken to prove unequivocally the identity of the assumed Tyr.Tyr spot. The spot was eluted and hydrolysed with 6N-HCl and found to yield tyrosine only. In addition, the ratio between the absorption at 2420 Å (in 0·1N-NaOH) and the intensity of the ninhydrin colour (at 5700 Å) was determined in the eluted intact material, and found to be close to the corresponding value for authentic Tyr_2. This number is a measure of the ratio between tyrosine residues and terminal amino groups in the substance tested. The ratios obtained from similar measurements carried out with tyrosine, Phe.Tyr and Glu.Tyr were, as expected, approximately half of that found for Tyr_2.

Closer inspection of the enzymic reaction at shorter time intervals gave a significant clue to the reaction mechanism. In this, as well as in the following experiments, advantage was taken of the fact that peptide derivatives containing the glutamic acid residue show rather large mobilities and good separations on paper electrophoresis (pH 6·8), that tyrosine and Tyr_2 separate sufficiently

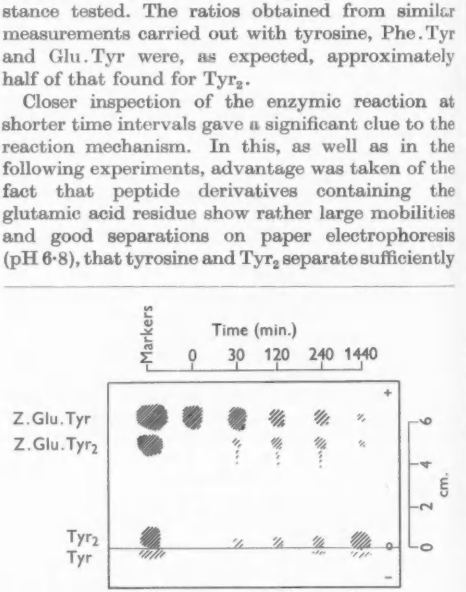


Fig. 1. Electrophoresis diagram showing the action of pepsin on benzyloxycarbonyl-L-glutamyl-L-tyrosine. The reaction mixture contained 13·5 mg. of Z.Glu.Tyr and 9 mg. of pepsin in 1 ml. of acetate buffer (pH 4·7, ionic strength 0·1), and was incubated at 37°. Spots were revealed by the diazo reagent. Time of electrophoresis: 2 hr.

well at this pH, the former moving to the cathode, the latter to the anode and that tyrosine-containing molecules can be specifically detected by the diazo reagent. An electrophoresis diagram of samples withdrawn from an enzymic reaction mixture at different times is shown in Fig. 1. It can be seen that the substrate (Z.Glu.Tyr) spot decreases steadily and the Tyr_2 spot increases. A small quantity of tyrosine is also formed. There appears, however, a new spot identified as Z.Glu.Tyr_2 by its mobility. It shows up clearly at the initial stages of the reaction, when almost no Tyr_2 is yet formed. It is present in a constant concentration while the amount of Tyr_2 is increasing, and disappears towards the end of the reaction together with the substrate (Z.Glu.Tyr). Z.Glu (detected as described in the Methods section), on the other hand, appeared already at the very start of the enzymic reaction, and its amount increased steadily with time.

The above results, where Z.Glu.Tyr yields Z.Glu and Tyr_2 as well as some Tyr, and where Z.Glu.Tyr_2 appears as an intermediate, might be explained by the following set of reactions:

(*a*) $2Z.Glu.Tyr \rightarrow Z.Glu.Tyr_2 + Z.Glu$

(*b*) $Z.Glu.Tyr \rightarrow Z.Glu + Tyr$

(*c*) $Z.Glu.Tyr_2 \rightarrow Z.Glu + Tyr_2$

The following experiment indicates, however, that the actual reaction mechanism is more complex.

N-Benzyloxycarbonyl-L-glutamyl-L-tyrosyl-L-tyrosine (Z.Glu.Tyr_2). This experiment was performed in order to clarify the behaviour towards pepsin of Z.Glu.Tyr_2, which appears as intermediate in the enzymic experiment with Z.Glu.Tyr. Comparison of the rate of peptic digestion of Z.Glu.Tyr_2 (pH 4·7) with that of Z.Glu.Tyr, under similar conditions, showed that the former is digested initially much more rapidly than the latter. Z.Glu.Tyr_2 yielded, at the early stages of enzymic incubation (1–2 hr.), mainly Tyr_2 and Z.Glu.Tyr, as detected electrophoretically (Fig. 2). After 2 hr. the amount of Z.Glu.Tyr formed already exceeds that of the remaining substrates. The latter stages of the reaction resemble therefore those observed in the enzymic experiment with Z.Glu.Tyr. The final reaction mixture was shown to contain Z.Glu, Tyr_2 and tyrosine.

As only traces of tyrosine appear at the beginning of the reaction the formation of Z.Glu.Tyr can be explained only by a transpeptidation reaction followed by hydrolysis:

$2\,Z.Glu.Tyr_2 \rightarrow Z.Glu.Tyr + Z.Glu.Tyr_3$

$Z.Glu.Tyr_3 \rightarrow Z.Glu.Tyr + Tyr_2$

The scheme suggested explains also the appearance, at the initial phase of the reaction, of relatively large amounts of Tyr_2 not accompanied by corresponding quantities of Z.Glu. The compound Z.Glu.Tyr_3 could be detected on the electrophoresis pattern in small quantitities as an intermediate of the enzymic reaction. The peptic digestion of this intermediate is described in the following experiment.

N-Benzyloxycarbonyl-L-glutamyltri-L-tyrosine (Z.Glu.Tyr_3). Electrophoretic analysis of the reaction products, carried out as in the previous cases (see Fig. 3), showed that digestion by pepsin proceeds rapidly, yielding initially Tyr_2, Z.Glu.Tyr and Z.Glu.Tyr_2, but no tyrosine. The first two substances are probably formed by the direct hydrolysis of the substrate. Direct hydrolysis can,

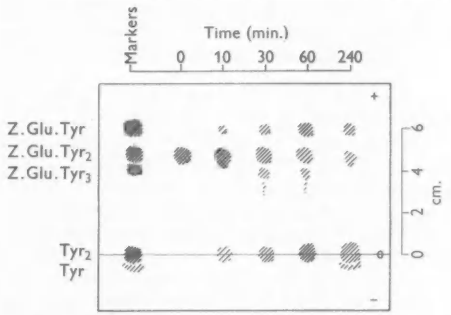


Fig. 2. Electrophoresis diagram showing the action of pepsin on benzyloxycarbonyl-L-glutamyl-L-tyrosyl-L-tyrosine. The reaction mixture contained 7·6 mg. of Z.Glu.Tyr_3 and 6·7 mg. of pepsin in 1 ml. of acetate buffer (pH 4·7, ionic strength 0·1), and was incubated at 37°. Spots were revealed by the diazo reagent. Time of electrophoresis: 2 hr.

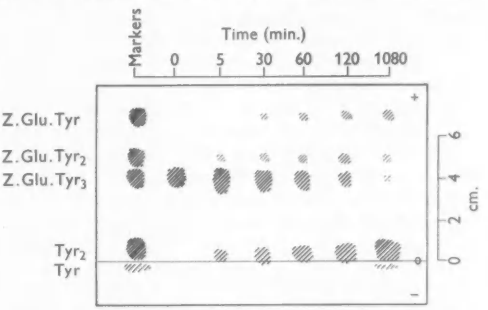


Fig. 3. Electrophoresis diagram showing the action of pepsin on benzyloxycarbonyl-L-glutamyl-L-trityrosine. The reaction mixture contained 6·3 mg. of Z.Glu.Tyr_3 and 4·3 mg. of pepsin in 1 ml. of acetate buffer (pH 4·7, ionic strength 0·1), and was incubated at 37°. Spots were revealed by the diazo reagent. Time of electrophoresis: 2 hr.

however, not be the cause of the formation of Z.Glu.Tyr_2, as no tyrosine was formed. Analogous to the case of Z.Glu.Tyr_2, a transpeptidation reaction involving Z.Glu.Tyr_4, yielding Z.Glu.Tyr_2 and Tyr_2 on hydrolysis, has to be assumed.

N-*Benzyloxycarbonyltri*-L-*tyrosine* (Z.Tyr_3). In this case, too, hydrolysis to Z.Tyr and Tyr_2 is fast. Transpeptidation, however, occurs here also to a certain extent, as is evident from the appearance of Z.Tyr_2, not accompanied by a parallel appearance of tyrosine.

N-*Benzyloxycarbonyl*-L-*phenylalanyl*-L-*tyrosine* (Z.Phe.Tyr). Analysis of the peptic digest (at pH 4·7) of this substrate was carried out similarly to that of Z.Glu.Tyr. Tyr and Tyr_2 (identified as above) were formed, but no spot corresponding to Phe.Tyr (R_F 0·80 in butanol–acetic acid–water) could be detected. Tyr_2 is obviously the result of a transpeptidation reaction followed by hydrolysis:

$$2\ \text{Z.Phe.Tyr} \rightarrow \text{Z.Phe.Tyr}_2 + \text{Z.Phe}$$

$$\text{Z.Phe.Tyr}_2 \rightarrow \text{Z.Phe} + \text{Tyr}_2$$

N-*Benzyloxycarbonyl*-L-*glutamyl*-L-*tyrosine amide* (Z.Glu.Tyr.Am). This substrate was hydrolysed in the presence of pepsin at pH 4·5 to yield Z.Glu and Tyr.Am, the latter giving the only ninhydrin-positive spot (R_F 0·79 in propanol–water–conc. aq. NH_3 soln.; R_F 0·45 in butanol–acetic acid–water) on a chromatogram of a 48 hr. peptic digest.

The absence of a transpeptidation reaction in the present case indicates that pepsin-catalysed transpeptidation reactions require an acceptor with a free α-carboxyl group.

Mixture of N-*benzyloxycarbonyl*-L-*tyrosyl*-L-*tyrosine* (Z.Tyr_2) *and benzyloxycarbonyl*-L-*glutamic acid* (Z.Glu). A reaction mixture consisting of Z.Tyr_2 (15 μmoles), Z.Glu (40 μmoles), pepsin (6 mg.), acetate buffer, pH 4·5 (0·9 ml.), and ethanol (0·1 ml.) was incubated at 37° for 2 days. In a control experiment the Z.Glu was omitted. Paper electrophoresis (2 hr.) of the control digest showed, with the diazo reagent, four constituents, located at −0·4 cm., +1·2 cm., +3·1 cm. and +4·5 cm. from the origin. By comparison with synthetic markers they were identified as Tyr, Tyr_2, Z.Tyr_2 and Z.Tyr respectively. With the peptic digest of the mixture containing Z.Glu an additional anionic diazo-positive spot was detected +6·5 cm. from the origin after electrophoresis for 2 hr. It was identified as Z.Glu.Tyr by comparison with a synthetic marker, and by the fact that on acid hydrolysis it gave rise to approximately equimolar quantities of tyrosine and glutamic acid. Z.Glu, which does not react with the diazo reagent, was detected with ninhydrin after treatment of the paper with HBr (see Methods section). At the end of 2 hr. it had travelled a distance of 9·5 cm. towards the anode, thus being clearly separated from Z.Glu.Tyr. No reaction occurred when Z.Glu and Tyr were incubated with pepsin.

The formation of the Z.Glu.Tyr may be formulated as follows:

$$\text{Z.Tyr.Tyr} + \text{Z.Glu} \rightarrow \text{Z.Tyr} + \text{Z.Glu.Tyr}$$

The fact that Z.Glu can serve as an acceptor for the terminal tyrosine moiety of Z.Tyr.Tyr proves the occurrence of a transpeptidation reaction of the amino transfer type.

Mixture of N-*benzyloxycarbonyl*-L-*phenylalanyl*-L-*tyrosine* (Z.Phe.Tyr) *and benzyloxycarbonyl*-L-*glutamic acid* (Z.Glu). A reaction mixture consisting of Z.Phe.Tyr (16 μmoles), Z.Glu (40 μmoles) pepsin (8 mg.), acetate buffer, pH 4·5 (0·9 ml.), and ethanol (0·1 ml.) was incubated for 1 day. In the control experiment Z.Glu was omitted. As in the preceding experiment, electrophoresis papers sprayed with the diazo reagent showed the appearance of Z.Glu.Tyr in the reaction mixture:

$$\text{Z.Phe.Tyr} + \text{Z.Glu} \rightarrow \text{Z.Phe} + \text{Z.Glu.Tyr}$$

A relatively large amount of the transpeptidation product (Z.Glu.Tyr) was formed within 1 day.

Mixture of N-*benzyloxycarbonyl*-L-*glutamyl*-L-*tyrosine* (Z.Glu.Tyr) *and* N-*benzyloxycarbonyl*-L-*tyrosine* (Z.Tyr). A reaction mixture consisting of Z.Glu.Tyr (18 μmoles), Z.Tyr (30 μmoles) and pepsin (6·5 mg.) in acetate buffer, pH 4·5 (1 ml.),

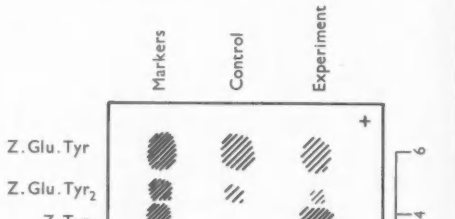


Fig. 4. Electrophoresis diagram showing the action of pepsin on a mixture of benzyloxycarbonyl-L-glutamyl-L-tyrosine and benzyloxycarbonyl-L-tyrosine. The reaction mixture contained 7 mg. of Z.Glu.Tyr, 9·5 mg. of Z.Tyr and 6·5 mg. of pepsin in 1 ml. of acetate buffer (pH 4·7, ionic strength 0·1), and was incubated at 37° for 24 hr. In the control experiment the Z.Tyr was omitted. Spots were revealed by the diazo reagent. Time of electrophoresis: 2 hr.

was incubated for 1 day. In the control experiment the Z.Tyr was omitted.

Electrophoretic analysis of the peptic digest showed a strong spot, which was identified as $Z.Tyr_2$ by its mobility (Fig. 4). The amounts of tyrosine and Tyr_2 formed were much smaller than in the control experiment. The formation of the $Z.Tyr_2$ is the result of a transpeptidation reaction in which Z.Tyr acts as acceptor:

$$Z.Glu.Tyr + Z.Tyr \rightarrow Z.Glu + Z.Tyr_2$$

Mixture of benzyloxycarbonyl-L-glutamyl-L-tyrosine (Z.Glu.Tyr) *and benzyloxycarbonylglycine* (Z.Gly). A similar experiment was made with Z.Gly as the acceptor and Z.Glu.Tyr as the tyrosine donor. After 2 days a new spot corresponding to that of Z.Gly.Tyr was located on an electrophoresis paper on spraying with the diazo reagent:

$$Z.Glu.Tyr + Z.Gly \rightarrow Z.Gly.Tyr + Z.Glu$$

Mixture of N-benzyloxycarbonyl-L-tyrosyl-L-tyrosine ($Z.Tyr_2$) *and N-benzyloxycarbonyl-L-phenylalanine* (Z.Phe). The reaction mixture consisted of $Z.Tyr_2$ (25 μmoles), Z.Phe (30 μmoles), pepsin (7·5 mg.), acetate buffer, pH 4·5 (0·5 ml.), and ethanol (0·1 ml.). It was incubated for 3 days at 37° before analysis. In the control experiment Z.Phe was omitted.

The expected formation of *N*-benzyloxycarbonyl-L-phenylalanyl-L-tyrosine (Z.Phe.Tyr), according to the scheme

$$Z.Tyr_2 + Z.Phe \rightarrow Z.Phe.Tyr + Z.Tyr,$$

could not be demonstrated by either chromatography or electrophoresis of the reaction mixture, since Z.Phe.Tyr cannot be separated by these techniques from $Z.Tyr_2$. The presence of Z.Phe.Tyr in the reaction mixture could, however, be shown by the isolation of Phe.Tyr after decarbobenzoxylation. The peptic digest was brought to dryness and the residue treated with 1 ml. of 30% (w/v) HBr in acetic acid. The precipitate formed on the addition of ether (20 ml.) was taken up in water (1 ml.) and analysed chromatographically with butanol–acetic acid–water as developer. In addition to the spots of tyrosine, Tyr_2 and phenylalanine (originating from excess of Z.Phe), with R_F values 0·45, 0·68 and 0·65 respectively, a spot with R_F 0·80, identical with that of authentic Phe.Tyr, appeared. The identity of the spot was confirmed by total hydrolysis, which gave rise to approximately equimolar quantities of tyrosine and phenylalanine.

DISCUSSION

In all the experiments with pepsin described in this paper, no 'energy-rich' compounds were present. The formation of new peptide bonds reported here may thus be explained only by a transpeptidation mechanism. The pepsin-catalysed transpeptidations could be demonstrated by the appearance of products with either an increased number of amino acid residues, or with a new amino acid sequence.

Elongation of the peptide chain was found with Z.Glu.Tyr and $Z.Glu.Tyr_2$, where, after short incubation with pepsin, $Z.Glu.Tyr_2$ and $Z.Glu.Tyr_3$ respectively were formed. The formation of new sequences was observed on peptic digestion of Z.Glu.Tyr and Z.Phe.Tyr, both of which yielded Tyr_2 as one of the final products. New sequences were also obtained when, in addition to the substrate, a suitable acceptor was present. The new sequences formed under these conditions are summarized in Table 1.

In some of the enzymic experiments products were formed which could have arisen by simple hydrolysis. Investigation of the other reaction products pointed, however, to a more complicated set of reactions. Thus $Ac.Tyr_2$ and $PAB.Tyr_2$ both gave a high yield of Tyr_2, but neither acetic acid nor *p*-aminobenzoic acid was formed. This led to the conclusion that the Tyr_2 must have been formed by the hydrolysis of an intermediate ($Ac.Tyr_3$ or $PAB.Tyr_3$) resulting from a transpeptidation reaction similar to the case of Z.Glu.Tyr, and not by a direct hydrolysis of the substrates.

An indirect argument for the occurrence of transpeptidation was used for $Z.Glu.Tyr_3$. Here the formation of $Z.Glu.Tyr_2$ in the first stages of the reaction was not accompanied by the corresponding amount of tyrosine. This indicated the formation of $Z.Glu.Tyr_4$ by the transpeptidation, followed by hydrolysis to $Z.Glu.Tyr_2$. In this case the intermediate $Z.Glu.Tyr_4$ could, however, not be clearly detected.

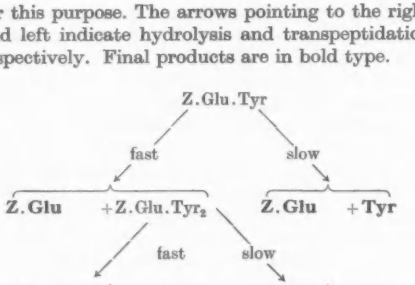
The possible complexity of the reactions taking place when certain low-molecular-weight peptides are digested by pepsin may be illustrated by the following reaction scheme describing the main hydrolysis and transpeptidation pathways in the peptic digestion of Z.Glu.Tyr. The scheme was constructed from the results obtained with Z.Glu.Tyr itself, as well as with the successive intermediates $Z.Glu.Tyr_2$ and $Z.Glu.Tyr_3$, synthesized

Table 1. *Products formed by a pepsin-catalysed transpeptidation in the presence of acceptors*

For explanation of abbreviation see Experimental section.

Substrate	Acceptor	Product
$Z.Tyr_2$	Z.Phe	Z.Phe.Tyr
$Z.Tyr_2$	Z.Glu	Z.Glu.Tyr
Z.Phe.Tyr	Z.Glu	Z.Glu.Tyr
Z.Glu.Tyr	Z.Tyr	$Z.Tyr_2$
Z.Glu.Tyr	Z.Gly	Z.Gly.Tyr

for this purpose. The arrows pointing to the right and left indicate hydrolysis and transpeptidation respectively. Final products are in bold type.



In the substrates investigated, the hydrolysis of the terminal peptide bond adjacent to a tyrosine residue bearing a free carboxyl group was slow as compared with transpeptidation. Fast enzymic hydrolysis was observed only in Z.Glu.Tyr_3 and Z.Tyr_3. The bond hydrolysed in these was the Tyr.Tyr bond not involving the *C*-terminal tyrosine. These findings recall similar observations of the behaviour of exo- and endo-peptide bonds of lysine peptides towards trypsin (Levin, Berger & Katchalski, 1956). With trypsin, as in the present case, digestion of Lys_2 and Lys_3, which are not hydrolysed readily, occurs via the synthesis of higher oligopeptides formed by a transpeptidation mechanism.

In the attempt to formulate the mechanism of the pepsin-catalysed transpeptidation discussed here, it was borne in mind that: (*a*) transpeptidation occurred only with substrates, such as Z.Glu.Tyr, Z.Phe.Tyr and Z.Tyr_2, possessing a free terminal α-carboxyl group; (*b*) blocking of the α-carboxyl group, as in Z.Glu.Tyr.Am, prevented transpeptidation; (*c*) all compounds acting as acceptors contained a free α-carboxyl group to which a tyrosine residue was transferred.

The overall reaction scheme thus involves the transfer of the tyrosine amino moiety of the donor $R^1{\cdot}CO{\cdot}NH{\cdot}R^2$ [R^2 = $CH(CO_2H){\cdot}CH_2{\cdot}C_6H_4{\cdot}OH$] to an acceptor with a free carboxyl group, $R^3{\cdot}CO_2H$:

$$R^1{\cdot}CO{\cdot}NH{\cdot}R^2 + R^3{\cdot}CO_2H \rightarrow R^3{\cdot}CO{\cdot}NH{\cdot}R^2 + R^1{\cdot}CO_2H,$$

and is therefore of the amino transfer type (Hanes *et al.* 1950). The detailed mechanism of the enzymic transfer and hydrolysis reactions might be envisaged as follows:

$$R^1{\cdot}CO{\cdot}NH{\cdot}R^2 + E{\cdot}OH \rightarrow R^1{\cdot}CO_2H + E{\cdot}HN{\cdot}R^2$$

$$E{\cdot}HN{\cdot}R^2 + R^3{\cdot}CO_2H \rightarrow R^3{\cdot}CO{\cdot}NH{\cdot}R^2 + E{\cdot}OH \quad \text{(transfer)}$$

$$E{\cdot}HN{\cdot}R^2 + H_2O \rightarrow H_2N{\cdot}R^2 + EOH \text{ (hydrolysis)}$$

The enzyme (E·OH) cleaves the susceptible bond of the substrate to yield an enzyme–tyrosine compound ($E{\cdot}HN{\cdot}R^2$), which is decomposed either by the acceptor ($R^3{\cdot}CO_2H$), with the formation of a new peptide bond, or by water, yielding free tyrosine.

Most of the transpeptidation reactions investigated occurred at about pH 4·5. This pH was chosen to facilitate solution of the substrates. It should be noted, however, that whenever tested it was found that pepsin could catalyse transpeptidation also at pH values as low as 1·8–2·0. In this low pH range, carboxyl groups are unionized. It appears therefore that it is the un-ionized carboxyl group that serves as the acceptor.

The possible existence of transpeptidation of the amino transfer type was pointed out by Hanes *et al.* (1950). It seems, however, that the present work constitutes the first experimental proof for such a reaction.

SUMMARY

1. The synthesis of the following compounds is described: *N*-*p*-nitrobenzoyl-L-tyrosine; *N*-*p*-nitrobenzoyl-L-tyrosine ethyl ester; *N*-*p*-nitrobenzoyl-L-tyrosine hydrazide; *N*-*p*-aminobenzoyl-L-tyrosine; L-tyrosyl-L-tyrosine ethyl ester hydrobromide; *N*-acetyl-L-tyrosyl-L-tyrosine; *NOO*-triacetyl-L-tyrosyl-L-tyrosine ethyl ester; *NO*-dibenzyloxycarbonyl-L-tyrosyl-L-tyrosine ethyl ester; *N*-*p*-nitrobenzoyl-L-tyrosyl-L-tyrosine; *N*-*p*-nitrobenzoyl-L-tyrosyl-L-tyrosine ethyl ester; *N*-*p*-aminobenzoyl-L-tyrosyl-L-tyrosine; L-phenylalanyl-L-tyrosine hydrobromide; *N*-benzyloxycarbonyl-L-phenylalanyl-L-tyrosine; tri-L-tyrosine ethyl ester hydrobromide; *NO*-dibenzyloxycarbonyl-L-tyrosyl-L-tyrosyl-L-tyrosine; *N*-benzyloxycarbonyl-L-glutamyl-L-tyrosyl-L-tyrosine; *N*-benzyloxycarbonyl-γ-benzyl-L-glutamyl-L-tyrosyl-L-tyrosine ethyl ester; *N*-benzyloxycarbonyl-L-glutamyltri-L-tyrosine and *N*-benzyloxycarbonyl-γ-benzyl-L-glutamyltri-L-tyrosine ethyl ester.

2. A method for the detection of *N*-benzyloxycarbonylamino acids on paper chromatograms was developed.

3. Acetyl-L-tyrosyl-L-tyrosine, *p*-aminobenzoyl-L-tyrosyl-L-tyrosine and *N*-benzyloxycarbonyl-L-tyrosyl-L-tyrosine yielded tyrosine and tyrosyltyrosine on peptic digestion. Evidence is presented to show that the tyrosyltyrosine was formed, not by a direct hydrolysis of the substrate but as a result

of a transpeptidation reaction, leading to the formation of an *N*-substituted trityrosine intermediate, followed by hydrolysis.

4. Peptic digestion of *N*-benzyloxycarbonyl-L-glutamyl-L-tyrosine led to the formation of tyrosine, tyrosyltyrosine and *N*-benzyloxycarbonyl-L-glutamic acid. *N*-Benzyloxycarbonylglutamyltyrosyltyrosine appeared as intermediate. A scheme describing the hydrolysis and transpeptidation reactions taking place in this case was constructed with the help of results obtained with *N*-benzyloxycarbonyl-L-glutamyl-L-tyrosyl-L-tyrosine and *N*-benzyloxycarbonyl-L-glutamyltri-L-tyrosine on digestion with pepsin.

5. Peptic digestion of *N*-benzyloxycarbonyl-L-phenylalanyl-L-tyrosine yielded tyrosine and tyrosyltyrosine, the latter being the result of a transpeptidation reaction.

6. Incubation of benzyloxycarbonyl-L-glutamyl-L-tyrosine amide with pepsin yielded *N*-benzyloxycarbonylglutamic acid and tyrosine amide. No transpeptidation was observed with this substrate.

7. The compounds *N*-benzyloxycarbonyl-L-phenylalanine, *N*-benzyloxycarbonyl-L-glutamic acid, *N*-benzyloxycarbonyl-L-tyrosine and *N*-benzyloxycarbonylglycine were shown to act as acceptors for the terminal tyrosine residue of the substrates, *N*-benzyloxycarbonyl-L-tyrosyl-L-tyrosine, *N*-benzyloxycarbonyl-L-glutamyl-L-tyrosine, acting as donors in the presence of pepsin.

8. Analysis of the pepsin-catalysed transpeptidation reaction showed that it involves the transfer of a tyrosine amino moiety of a donor to an acceptor with a free carboxyl group. It may thus be classified as a transpeptidation of the amino transfer type.

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REFERENCES

Baker, L. E. (1951). *J. biol. Chem.* **193**, 809.
Barkdoll, A. E. & Ross, W. F. (1944). *J. Amer. chem. Soc.* **66**, 951.
Ben-Ishai, D. & Berger, A. (1952). *J. org. Chem.* **17**, 1564.
Bergmann, M. & Zervas, L. (1932). *Ber. dtsch. chem. Ges.* **65**, 1192.
Bergmann, M., Zervas, L., Salzmann, L. & Schleich, H. (1934). *Hoppe-Seyl. Z.* **224**, 17.
Blau, K. & Waley, S. G. (1954). *Biochem. J.* **57**, 538.
Casey, E. J. & Laidler, K. J. (1950). *J. Amer. chem. Soc.* **72**, 2159.
Consden, R. & Stanier, W. M. (1952). *Nature, Lond.*, **170**, 1069.
Cramer, F. (1955). *Paper Chromatography*, p. 80. London: Macmillan and Co. Ltd.
Dalgliesh, C. E. (1952). *Biochem. J.* **52**, 3.
Dekker, C. A., Taylor, S. P., jun. & Fruton, J. S. (1949). *J. biol. Chem.* **180**, 155.
du Vigneaud, V. & Meyer, C. E. (1932). *J. biol. Chem.* **98**, 295.
Fruton, J. S. & Bergmann, M. (1939). *J. biol. Chem.* **127**, 627.
Hanby, W. E., Waley, S. G. & Watson, J. (1950). *J. chem. Soc.* p. 3245.
Hanes, C. S., Hird, F. J. R. & Isherwood, F. A. (1950). *Nature, Lond.*, **166**, 288.
Harington, C. R. & Pitt-Rivers, R. V. (1944). *Biochem. J.* **38**, 417.
Hutchens, J. O. & Kass, B. M. (1949). *J. biol. Chem.* **177**, 571.
Katchalski, E. & Sela, M. (1953). *J. Amer. chem. Soc.* **75**, 5824.
Kennedy, E. P. & Barker, H. A. (1951). *Analyt. Chem.* **23**, 1033.
Levin, Y., Berger, A. & Katchalski, E. (1956). *Biochem. J.* **63**, 308.
Vaughan, J. R., jun. & Osato, R. L. (1952). *J. Amer. chem. Soc.* **74**, 676.

Biochemical Adaptation as a Response to Exercise

1. EFFECT OF SWIMMING ON THE LEVELS OF LACTIC DEHYDROGENASE, MALIC DEHYDROGENASE AND PHOSPHORYLASE IN MUSCLES OF 8-, 11- AND 15-WEEK-OLD RATS

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Although adaptation to physical training has received extensive study from the aspects of performance and physiological effects (Åstrand, 1956), there is little information on this effect at the cellular level. It is significant that the comprehensive review of enzyme adaptation by Knox, Auerbach & Lin (1956) makes no reference whatever to enzymic changes accompanying athletic training.

Most of the published work in this field stems from the Leningrad Physical Culture Research Institute (Yakovlev, 1950; Yampolskaya & Yakovlev, 1951; Yampolskaya, 1952). These workers claim that muscle analyses after training have

shown increases in the concentrations of glycogen, phosphagen, ascorbic acid and glutathione, and increases in the 'phosphorolytic activity' and in the activity of hexokinase, lactic dehydrogenase and succinic dehydrogenase. On the other hand, Hearn & Wainio (1956) found no change in the concentration of succinic dehydrogenase in both heart and skeletal muscle of rats subjected to a more strenuous exercise regimen than that for those of the Russian workers. They concluded that the concentration of this enzyme was normally sufficient to cope with all demands on the animal.

The present paper is the first report of studies being undertaken in this Laboratory to explore further the effects of training. Lactic dehydrogenase, malic dehydrogenase and phosphorylase were chosen as enzymes of importance in the energy-producing system of rat muscle. In addition we have extended the work to include the possible effects of growth and maturity upon the adaptive response.

In their review Knox *et al.* (1956) stress the importance of the reference base when studying adaptation. Considering that a change in activity measured relative to the protein mass constitutes enzymic adaptation, we have selected non-collagen nitrogen as the most satisfactory basis in these initial tests.

EXPERIMENTAL

Materials

Distilled water prepared in all-glass apparatus was used throughout. Reduced coenzyme I (DPNH) was from Nutritional Biochemicals Corp., U.S.A. Pyruvic acid (British Drug Houses Ltd.) was triply distilled under reduced pressure. Oxaloacetic acid was synthesized according to the method of Wohl & Oesterlin (1901) and stored at −15°. Immediately before use a 0·03 M-solution in 0·06 M-$NaHCO_3$ was prepared. Cysteine hydrochloride and glycogen were from British Drug Houses Ltd., glucose 1-phosphate (dipotassium salt) from L. Light and Co. Ltd. and adenosine 5′-phosphate from Roche Products Ltd. All solutions were stored at −15°.

Methods

Male rats of the Wistar strain were used. One week before experiments began, litter mates were sorted into two groups of approximately equal weights, each group comprising nine animals. Food and water were freely available throughout the experiments. The animals were fed on the normal colony diet, a commercial cube preparation (Barastoc Products, Melbourne, Australia) having the following stated percentage composition: carbohydrate, 50; protein, 24; fat, 7; salts, 1; crude fibre, 4; moisture, 10; and including supplements to provide an adequate vitamin supply.

Three series of experiments were conducted on animals 8, 11 and 15 weeks old. As one animal of the second series died after earmarking, its corresponding control was removed, reducing both groups to eight animals. At their specified ages animals of the experimental groups commenced a programme of exercise consisting in swimming for 30 min. daily, for six 5-day weeks, in water at 27–30°. Body weights were recorded each week. Twenty-four hours after completing their exercise programme the animals were stunned by a blow on the head and decapitated. Muscle was quickly removed from the biceps region of the hind leg and chilled in ice. A '1:50' homogenate was prepared by slicing about 400 mg. of tissue thinly with a scalpel and blending it in 50 vol. of water in a Potter-Elvehjem apparatus (Potter & Elvehjem, 1936). Care was taken to ensure uniformity of sampling when pipetting such homogenates.

Lactic dehydrogenase assay. The spectrophotometric method of Wroblewski & La Due (1955) was employed, with a Beckman DU spectrophotometer. However, to ensure adequate substrate levels it was necessary to increase the concentration of sodium pyruvate in the cuvette to 3·3 mM. A portion (5·0 ml.) of the 1:50 homogenate was diluted to 250 ml. and 0·1 ml. of this dilute homogenate was incubated at 25° in the cuvette with 0·25 ml. of DPNH solution (1 mg./ml.) and 2·45 ml. of 0·05 M-phosphate buffer, pH 7·2 (28 vol. of 0·05 M-KH_2PO_4 + 72 vol. of 0·05 M-K_2HPO_4). After 20 min. the reaction was started by the addition of 0·2 ml. of 0·05 M-sodium pyruvate, pH 7·2, and the decrease in extinction at 340 mμ followed at 1 min. intervals. One dehydrogenase unit is defined as the amount of enzyme which brings about a decrease in extinction of 0·001/min. under the above conditions; the rate of change in extinction is proportional to the concentration of enzyme for changes in extinction less than 0·04/min.

Malic dehydrogenase assay. A spectrophotometric method adapted from that of Ochoa (1955) was employed. The assay differed from that described above for lactic dehydrogenase only in that 0·2 ml. of 1:2500 homogenate was assayed and 0·25 ml. of 0·03 M-sodium oxaloacetate served as the substrate. The final volume of contents in the cuvette was again 3·0 ml.; the rate of change in extinction is proportional to the concentration of enzyme if the rate does not exceed 0·025/min.

Phosphorylase assay. The method of Illingworth & Cori (1953) was employed with slight modifications as follows: 5·0 ml. of the 1:50 homogenate was mixed with 10·0 ml. of 0·08 M-sodium glycerol β-phosphate buffer, pH 6·8, containing NaF at a concentration of 0·4 M. A portion (2·0 ml.) of this enzyme–buffer mixture was diluted with an equal volume of a freshly neutralized (pH 6·8) solution of 0·06 M-cysteine. A portion (0·5 ml.) of this suspension was incubated at 30° with 0·3 ml. of 3·3% (w/v) glycogen for 10 min., and the reaction started by the addition of 0·2 ml. of solution containing 64 mM-glucose 1-phosphate and 5 mM-adenylic acid (pH 6·8). At 5, 10 and 15 min., samples (0·2 ml.) were taken and assayed for inorganic phosphate by the method of Fiske & Subbarow (1925), in an EEL (Evans Electroselenium Ltd.) photoelectric colorimeter with the red OR1 filter. Activity was expressed in the units of Illingworth & Cori (1953).

Non-collagen nitrogen. Non-collagen nitrogen was separated by the method of Lilienthal, Zierler, Folk, Buka & Riley (1950). The alkali extract was digested according to the method of Chibnall, Rees & Williams (1943) and the ammonia in the digest estimated, with the micro-still described by Archibald (1943). The details were as follows:

5·0 ml. of 1:50 homogenate was mixed with 50 ml. of 0·05 N-NaOH, left overnight at 21° and filtered. A portion (5·0 ml.) of this extract was digested on an electrically-heated rack overnight with 2 ml. of concentrated H_2SO_4 and 0·5 g. of selenium catalyst. The digest was diluted to 50 ml., 5·0 ml. samples were neutralized in the still with 2·5 ml. of 4 N-NaOH and, after a 6 min. distillation, the distillate was diluted to 40 ml. To 10 ml. samples, 0·5 ml. of Nessler's solution was added and, after 5 min., the colour read in the EEL photoelectric colorimeter with the blue OB 10 filter.

RESULTS

Table 1 lists details of initial weights and weight gains. In all cases the exercised animals gained considerably less weight than did their corresponding controls. This weight-difference appeared as early as 1 week after exercise had commenced and was maintained throughout the experiment in all the age groups.

The results of the enzyme assays are shown in Table 2. If the enzyme activities of the exercised groups are compared with those of the controls, in only two cases—the lactic dehydrogenase levels of the 8-week-old series and the phosphorylase of the 15-week-old series—are statistically significant differences shown. In both these cases the degree of significance ($P < 0·05$) is considered insufficient to constitute a positive effect. Similarly, no differences were found between the corresponding groups of the three series.

DISCUSSION

The results are in agreement with those of Hearn & Wainio (1956), both as regards weight changes and the absence of any change in enzyme concentration. That this absence of change is not merely an indication of an inadequate exercise programme is shown by the weight differences induced (Hearn & Wainio, using identical exercise, also demonstrated increased weights of heart ventricle and adrenal) and the fact that the training programme employed by the Russian workers (Yakovlev, 1950) was less strenuous than that used in this study. The animals in the Russian experiments were exercised for 30 days by swimming for 1 min. on the first day, this increasing by 1 min./day to a total of 30 min. on the final day.

The cause of the disparity between the Russian results, on the one hand, and those of Hearn & Wainio and ourselves, on the other, would appear to be the difference in assay methods employed. The techniques of short-duration, optimum-condition assay described above result in activities which are proportional to the concentration of enzymes studied. The techniques used in the Russian experiments involve long incubations with relatively large samples of tissue brei. It is doubtful whether the activities so obtained were proportional to enzyme concentration, but would depend rather on the presence of endogenous substrates and other

Table 1. *Average initial weights and weight gains of male Wistar rat control groups and groups exercised by swimming 30 min. daily for 6 weeks*

Age (weeks)	Group	No. of animals	Initial wt. (g.) (mean ± S.E.)	Wt. gain (g./week) (mean ± S.E.)
8	Exercised	9	193 ± 16	9·9 ± 2·7
	Control	9	191 ± 19	16·3 ± 3·7
11	Exercised	8	274 ± 15	8·6 ± 3·1
	Control	8	272 ± 20	12·7 ± 3·6
15	Exercised	9	316 ± 21	3·7 ± 1·3
	Control	9	309 ± 35	8·6 ± 3·3

Table 2. *Enzyme levels in skeletal muscle of male Wistar rats*

For details and definition of units see Methods.

Age at start of experiment (weeks)	Group	No. of animals	Lactic dehydrogenase (mean ± S.E.†)	Malic dehydrogenase (mean ± S.E.†)	Phosphorylase (mean ± S.E.‡)
8	Exercised	9	13·7 ± 7·0*	6·1 ± 1·4	128·0 ± 14·6
	Control	9	10·7 ± 4·0	5·1 ± 2·5	123·6 ± 11·3
11	Exercised	8	12·5 ± 4·8	4·5 ± 1·3	123·0 ± 10·5
	Control	8	12·2 ± 4·2	5·3 ± 2·1	125·8 ± 15·2
15	Exercised	9	13·8 ± 4·3	4·9 ± 1·1	132·0 ± 9·2*
	Control	9	12·1 ± 4·4	4·8 ± 1·4	122·1 ± 9·1

* Exercised *vs.* control: $P < 0·05$.
† Units (thousands)/mg. of non-collagen nitrogen.
‡ Units/mg. of non-collagen nitrogen.

substances in the tissue samples, which might act as activators or inhibitors. Such activities would reflect more the overall metabolic potential of the disrupted muscle. Here perhaps lie the reasons for the apparent contradiction in results. For while the Russian results indicate that the metabolic potential of muscle has been enhanced by training, thus demonstrating biochemically a phenomenon which has been well defined physiologically, the concentrations of enzymes intimately concerned with the utilization of carbohydrate energy sources remain unaltered. The metabolic activity of the muscle would then depend on the mobilization of the enzyme systems by control mechanisms. Yampolskaya (1952) has shown that the 'phosphorolytic activity' (that is the hexose phosphate produced from glycogen after a 2 hr. incubation at 37°) of muscle taken from trained rats is considerably greater than that from untrained animals. After the rats had swum for 3 min. both values were increased. When the animals were anaesthetized with Amytal, thus depressing the effect of the central nervous system, the resting value for the trained animal fell to a figure only slightly greater than that of the control (which remained unaltered). After the anaesthetized animals had undergone electrically-stimulated exercise, similar in intensity to the 3 min. swim, the values for both trained and untrained rats were only slightly elevated. These results indicate the extent to which control mechanisms may be responsible for the activity increases reported by the Russian workers. Thus the adaptive responses would appear to involve regulatory mechanisms rather than any change in enzyme levels.

SUMMARY

1. Male rats (Wistar strain) 8, 11 and 15 weeks old were exercised by swimming 30 min. daily for six five-day weeks.

2. The exercised animals gained significantly less weight than their control groups. Muscle assays revealed no differences in the levels of lactic dehydrogenase, malic dehydrogenase and phosphorylase, either between exercised or control groups or between the three different age groups.

3. While other workers have shown that training enhances the metabolic activity of muscle, the constancy of enzyme levels reported here suggests that the adaptive response involves other controlling factors.

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REFERENCES

Archibald, R. M. (1943). *J. biol. Chem.* **151**, 141.
Åstrand, P. O. (1956). *Physiol. Rev.* **36**, 307.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Hearn, G. R. & Wainio, W. W. (1956). *Amer. J. Physiol.* **185**, 348.
Illingworth, B. & Cori, G. T. (1953). *Biochem. Prep.* **3**, 1.
Knox, W. E., Auerbach, V. H. & Lin, E. C. C. (1956). *Physiol. Rev.* **36**, 164.
Lilienthal, J. L., jun., Zierler, K. L., Folk, B. P., Buka, R. & Riley, M. J. (1950). *J. biol. Chem.* **182**, 501.
Ochoa, S. (1955). In *Methods in Enzymology*, vol. 1, p. 735. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Wohl, A. & Oesterlin, C. (1901). *Ber. dtsch. chem. Ges.* **34**, 1139.
Wroblewski, F. & La Due, J. S. (1955). *Proc. Soc. exp. Biol., N.Y.*, **90**, 210.
Yakovlev, N. N. (1950). *Sechenov J. Physiol.* **36**, 744.
Yampolskaya, L. I. (1952). *Sechenov J. Physiol.* **38**, 91.
Yampolskaya, L. I. & Yakovlev, N. N. (1951). *Sechenov J. Physiol.* **37**, 110.

Biochemical Adaptation as a Response to Exercise

2. ADENOSINE TRIPHOSPHATASE AND CREATINE PHOSPHOKINASE ACTIVITY IN MUSCLES OF EXERCISED RATS

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In continuation of our previous work (Gould & Rawlinson, 1959), the effect of training on two of the enzymes concerned with the process of muscular contraction, adenosine triphosphatase and creatine phosphokinase, was studied. As both of these enzymes require calcium for maximum activity, availability of Ca^{2+} ions might serve as a means of regulating their activity [DuBois & Potter (1943) suggest that this is true for adenosine triphosphatase], and so the activities of these enzymes

were assayed both with optimum amounts of added calcium and in the absence of added calcium.

As increases in the metabolic activity of muscles of both trained and untrained rats, after they had swum for from 1 to 15 min., have been reported (Yampolskaya, 1952; Chagovets, 1957), we have included the effects of such exercise in this study. This is referred to below as the 'short exercise' effect to distinguish it from the effect of the 6 weeks' training period.

Hearn & Wainio (1957) reported small but significant increases in the aldolase activity of heart and skeletal muscle of trained rats, the activity being expressed on a wet-weight basis. However, these authors also found similar increases in the protein content of the wet tissue; so the aldolase activity, when expressed relative to the protein concentration, as in their earlier report (Hearn & Wainio, 1956), would appear to have been unaltered by training. This result stresses the importance of the basis of calculation in such studies. In accordance with the criterion for enzymic adaptation adopted in the preceding paper (Gould & Rawlinson, 1959), i.e. that the enzymic activity should be measured relative to the protein mass, we have again expressed activities relative to non-collagen nitrogen. In addition, determinations of wet and dry weight and total nitrogen have been included.

EXPERIMENTAL

Materials

Sodium phosphocreatine and *N*-ethylmorpholine were gifts from Dr H. Rosenberg. Adenosine diphosphate and adenosine triphosphate (sodium salts) were obtained from the Sigma Chemical Co., U.S.A., and barbitone sodium (B.P.) from May and Baker Ltd. For M-$CaCl_2$, the calculated amount of $CaCO_3$ was neutralized with 5N-HCl, and the solution was boiled and diluted to volume. *p*-Chloromercuribenzoic acid (L. Light and Co. Ltd.) was recrystallized according to Whitmore & Woodward (1941) and dissolved in warm 5N-NaOH. Conc. HCl was added until a faint white precipitate persisted; this was cleared with a few drops of 5N-NaOH and the solution of the sodium salt diluted to the required volume. Creatine (British Drug Houses Ltd.) was recrystallized according to Hunter (1928). For the creatine colour reagent, a stock solution of diacetyl was prepared from dimethylglyoxime (A.R., By-Products and Chemicals Pty. Ltd., Sydney) as described by Walpole (1911). The distillate (50 ml.) was diluted to 100 ml. to give an approx. 1% solution of diacetyl (Eggleton, Elsden & Gough, 1943). α-Naphthol (5 g.) was dissolved in 20 ml. of propan-1-ol, 2·5 ml. of 1% diacetyl was added and the volume adjusted to 100 ml. with propan-1-ol. Cysteine hydrochloride was laboratory-reagent grade (British Drug Houses Ltd.). Myosin was prepared according to Bailey (1942). After three precipitations, the myosin–KCl solution was mixed with an equal volume of glycerol and stored at −15°. α-Naphthol (British Drug Houses Ltd.) was purified by steam distillation; the dried crystals were stored in a dark bottle at −15°. Propan-1-ol (British Drug Houses Ltd.) was purified by distillation after refluxing it for 1 hr. over NaOH and Al powder.

Methods

Two series of experiments were conducted with male rats of the Wistar strain, 8 weeks old at the start of the experiment. Litter mates of similar weights were divided into four groups (*A*, *B*, *C*, *D*). Food and water were freely available throughout the experiments. The animals of groups *C* and *D* were trained by swimming for 30 min. daily for six 5-day weeks in water at 27–30°; those of groups *A* and *B* were accustomed to water by being placed in the exercise tank for 20 sec. each day. Twenty-four hours after completing the exercise programme the animals of groups *A* and *C* were killed at rest while those of groups *B* and *D* were first exercised. In the first series each group contained 9 animals and the short exercise consisted of swimming for 5 min. In the second series there were 10 animals in each group, while the short-exercise period was increased to 30 min. Muscle homogenates were prepared as before (Gould & Rawlinson, 1959). Those from the animals of the first series were assayed for adenosine triphosphatase (ATPase) and creatine phosphokinase (CPK), while only ATPase was assayed in the second series. In the second series, two samples of muscle (approximately 1 g.) were taken from each animal and weighed. On one sample dry weight was determined after heating for 21 hr. at 105°, while the other was homogenized in water and diluted to 50 ml. A portion (5 ml.) of this homogenate was mixed with 20 ml. of 0·05N-NaOH and incubated overnight at 21°. Total nitrogen was assayed by digestion of 5·0 ml. of this suspension; the remainder was filtered and non-collagen nitrogen determined by digestion of 5·0 ml. of filtrate. The digestion and NH_3 determination were as previously described (Gould & Rawlinson, 1959), except that the digest was diluted to 100 ml. and the distillate to 20 ml., followed by the addition of 1·0 ml. of Nessler's solution to the entire distillate. Non-collagen nitrogen in the homogenates used in the enzyme studies was determined by the above procedure.

Adenosine triphosphatase. ATPase was determined by the method of DuBois & Potter (1943). As the precipitated protein has little effect on the colorimeter reading, and is compensated for in the enzyme blank, it was unnecessary to centrifuge the reaction mixture after the addition of trichloroacetic acid; the method was modified to enable the entire procedure to be carried out in the one tube. Instead of duplicates, the homogenate was assayed at dilutions of 1:100 and 1:200, thus making possible a continuous check on linearity.

Homogenate (0·1 ml.) was incubated with 0·2 ml. 6 mM-$CaCl_2$–25 mM-barbiturate buffer (pH 7·4), for 5 min. at 37° in tubes calibrated at 10 ml. The reaction was started by the addition of 0·1 ml. of 12 mM-ATP, pH 7·4, previously brought to 37°, and after 15 min. stopped with 0·1 ml. of ice-cold 25% trichloroacetic acid. Molybdate I (1·0 ml.) and aminonaphtholsulphonic acid reagents (0·4 ml.) (Fiske & Subbarow, 1925) were added and the volume adjusted to 10 ml. with water. The colour was read after 15 min. in an EEL (Evans Electroselenium Ltd.) photoelectric colorimeter with the red OR1 filter. Blanks to determine the inorganic phosphorus present in the reaction mixture

and homogenate were included. The activity was also determined in the absence of added calcium. One ATPase unit is defined as the amount of ATPase which liberates 1 μg. of inorganic phosphorus in 15 min. at 37°.

Creatine phosphokinase. This was determined by using a modification of the method of Ennor & Stocken (1948), as advised by Dr H. Rosenberg (personal communication). A stock reaction mixture was prepared daily, comprising 1·0 ml. of 25 mM-cysteine hydrochloride (neutralized with 1 drop of M-*N*-ethylmorpholine), 1·0 ml. of 0·5 M-*N*-ethylmorpholine buffer (pH 7·2), 0·4 ml. of 0·1 M-$CaCl_2$, 0·5 ml. of 10 mM-ADP (pH 7·2), 0·5 ml. of myosin solution and water to 5·0 ml. The tissue was assayed at dilutions of 1:10 000 and 1:20 000 and, in the absence of added calcium, at the 1:500 level, all dilutions being made with distilled water.

Diluted homogenate (1·0 ml.) and stock reaction mixture (0·5 ml.) were equilibrated at 37° for 5 min. The reaction was started by the addition of 0·5 ml. of 2 mM-sodium phosphocreatine (pH 7·5) and, after a 3 min. incubation, stopped by the addition of 2 ml. of a solution containing 2 vol. of 0·2 M-ethylenediaminetetra-acetic acid, 3 vol. of 5 N-NaOH and 5 vol. of 20 mM-sodium *p*-chloromercuribenzoate. Creatine colour reagent (2 ml.) was added and the volume adjusted to 10 ml. with water. The colour was read after 15 min. on the EEL photoelectric colorimeter with the green OGR1 filter, and compared against a standard containing 10 μg. of creatine. The standard solution, stored at −15°, was never kept for longer than 1 week. Blanks to determine the creatine present in the reaction mixture and homogenate were included. One CPK unit is defined as the amount of enzyme which liberates 1 μg. of creatine under the conditions described above. Maximum activity is not attained with the above concentration of phosphocreatine (0·5 mM), but a linear activity–enzyme relationship exists, and on the grounds of economy the use of this concentration was preferable.

Table 1. *Adenosine triphosphatase and creatine phosphokinase levels in the muscles of male Wistar rats, trained and untrained, at rest and immediately after a 5 min. swim*

For details and definition of units see Methods.

Enzyme (units/mg. of non-collagen nitrogen)	Activity	Untrained (mean of 9 animals ± S.E.) Rest	Untrained (mean of 9 animals ± S.E.) Swim 5 min.	Trained (mean of 9 animals ± S.E.) Rest	Trained (mean of 9 animals ± S.E.) Swim 5 min.
ATPase	Total	630 ± 105	632 ± 110	591 ± 87	592 ± 128
	Measured in absence of added Ca^{2+}	458 ± 57	445 ± 123	422 ± 56	425 ± 64
CPK	Total	9800 ± 1600	10 300 ± 1900	9600 ± 2200	9400 ± 1700
	Measured in absence of added Ca^{2+}	374 ± 106	384 ± 159	345 ± 131	416 ± 93

Table 2. *Adenosine triphosphatase levels in the muscles of male Wistar rats, trained and untrained, at rest and immediately after a 30 min. swim*

For details and definition of units see Methods. The ATPase is expressed as units/mg. of non-collagen nitrogen.

Activity	Control (mean of 10 animals ± S.E.) Rest	Control (mean of 10 animals ± S.E.) Swim 30 min.	Trained (mean of 10 animals ± S.E.) Rest	Trained (mean of 10 animals ± S.E.) Swim 30 min.
Total	480 ± 62	502 ± 93	508 ± 88	542 ± 87
Measured in absence of added Ca^{2+}	313 ± 32	323 ± 53	315 ± 52	349 ± 75

Table 3. *Dry weight, total nitrogen and non-collagen nitrogen contents of the muscles of male Wistar rats, trained and untrained, at rest and immediately after a 30 min. swim*

For details see Methods.

	Control (mean of 10 animals ± S.E.) Rest	Control (mean of 10 animals ± S.E.) Swim 30 min.	Trained (mean of 10 animals ± S.E.) Rest	Trained (mean of 10 animals ± S.E.) Swim 30 min.
Dry weight (mg./g. wet wt.)	259·0 ± 9·5	255·7 ± 17·4	255·2 ± 7·9	253·0 ± 13·9
Total nitrogen (mg./g. wet wt.)	37·5 ± 2·1	37·2 ± 2·2	37·9 ± 2·4	37·8 ± 3·0
Total nitrogen (mg./g. dry wt.)	144·9 ± 6·6	147·2 ± 13·8	148·7 ± 10·0	149·5 ± 15·4
Non-collagen nitrogen (mg./g. wet wt.)	35·2 ± 2·2	35·2 ± 1·9	35·8 ± 2·6	35·7 ± 2·6
Non-collagen nitrogen (mg./g. dry wt.)	135·8 ± 8·1	139·4 ± 12·8	140·6 ± 10·8	141·1 ± 13·8

RESULTS

The levels of ATPase and CPK in trained and untrained animals, both at rest and immediately after swimming for 5 min., are shown in Table 1. These results were subjected to statistical analysis by the method of analysis of variance, which showed no differences between either trained or untrained groups of animals, at rest or after the short exercise. Similarly, the activities of these enzymes when determined in the absence of added calcium showed no differences.

In the second series (Table 2), where the short exercise had been increased to 30 min., the activity of ATPase was significantly increased by training and also by the short exercise. The activity of the enzyme in trained animals was 2·6 % higher than that of the untrained, and that of the animals which had swum for 30 min. was 2·8 % higher than the corresponding group killed at rest; in all cases the differences were significant at the 5 % level. The physiological significance of such small increases is slight. The activity determined in the absence of added calcium paralleled the total activity in all cases.

From Table 3 it will be seen that none of the reference bases showed any effect of either training or short exercise.

DISCUSSION

The adaptation of metabolic processes to training could occur in the following possible ways: There could be (*a*) no change in the enzymic pattern of the muscle, the increased performance being due to the observed hypertrophy of the tissue (Steinhaus, 1933); (*b*) no change in the enzymic pattern of the muscle as far as the relative concentration of enzymes is concerned, but changes which result in greater activity of these enzymes; (*c*) changes involving the relative concentrations of enzymes, with or without accompanying changes in their activity. As hypertrophy of skeletal muscle does not always accompany athletic training (Steinhaus, Hoyt & Rice, 1932; Hearn & Wainio, 1956), and in view of the increased metabolic activities reported by the Russian workers, it would appear that simple hypertrophy cannot by itself constitute the adaptive response.

Nor need the response necessarily involve changes in enzyme concentrations, as indeed has been demonstrated by the above results and those previously reported by both Hearn & Wainio (1956, 1957) and ourselves (Gould & Rawlinson, 1959). As the enzyme systems *in vivo* are probably operating at rates considerably below maximum, greater activity of the tissue could be achieved by increasing these rates rather than increasing the concentration of the enzymes involved. The absence of any marked changes in the concentrations of either ATPase or CPK as a result of the training programme is in agreement with previous results where lactic dehydrogenase, malic dehydrogenase, phosphorylase (Gould & Rawlinson, 1959), succinic dehydrogenase (Hearn & Wainio, 1956) and aldolase (Hearn & Wainio, 1957) were studied. The determinations of ATPase and CPK in the absence of added Ca^{2+} have shown that though this ion is required by these two enzymes, their activity under the above conditions does not seem to have been regulated by its availability.

The absence of any changes in the concentrations of ATPase after the 5 or 30 min. swim, or of CPK after the 5 min. swim, in contrast with the increased activity reported by Yampolskaya (1952), may be due to differences in analytical methods (see Gould & Rawlinson, 1959).

These results indicate that adaptation to exercise is not characterized by changes in the levels of the important muscle enzymes investigated. Although it is possible that some other enzyme(s) might be limiting we feel that further work would be more fruitfully directed towards regulatory mechanisms, in particular the uptake of substrates by the muscle cell.

SUMMARY

1. Male Wistar rats, 8 weeks old, were exercised by swimming for 30 min. daily for six 5-day weeks. The control animals were placed in the tank for 20 sec. daily to accustom them to the water.

2. Adenosine triphosphatase and creatine phosphokinase were assayed in the muscles of the trained animals and their controls, both at rest and immediately after swimming for 5 min. Adenosine triphosphatase was also determined after a 30 min. swim.

3. Neither the total activity of the two enzymes nor the activity measured in the absence of added calcium was affected by either the training or short-exercise procedures.

4. The dry weight, total nitrogen and non-collagen nitrogen contents of the muscle were similarly unaffected by either the training or short exercise.

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REFERENCES

Bailey, K. (1942). *Biochem. J.* **36**, 121.
Chagovets, N. R. (1957). *Ukr. biochem. J.* **29**, 450.
DuBois, K. P. & Potter, V. R. (1943). *J. biol. Chem.* **150**, 185.
Eggleton, P., Elsden, S. R. & Gough, N. (1943). *Biochem. J.* **37**, 526.
Ennor, A. H. & Stocken, L. A. (1948). *Biochem. J.* **42**, 557.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Gould, M. K. & Rawlinson, W. A. (1959). *Biochem. J.* **73**, 41.
Hearn, G. R. & Wainio, W. W. (1956). *Amer. J. Physiol.* **185**, 348.
Hearn, G. R. & Wainio, W. W. (1957). *Amer. J. Physiol.* **190**, 206.
Hunter, A. (1928). *Creatine and Creatinine.* London: Longmans, Green and Co.
Steinhaus, A. H. (1933). *Physiol. Rev.* **13**, 103.
Steinhaus, A. H., Hoyt, L. A. & Rice, H. A. (1932). *Amer. J. Physiol.* **99**, 512.
Walpole, G. S. (1911). *J. Physiol.* **42**, 301.
Whitmore, F. C. & Woodward, G. E. (1941). *Org. Synth.* (*Coll.*), 2nd ed. **1**, 159.
Yampolskaya, L. I. (1952). *Sechenov J. Physiol.* **38**, 91.

Enzymes of the Pentose Phosphate Pathway in the Mung-Bean Seedling

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It is well known that glucose 6-phosphate, apart from undergoing anaerobic breakdown by the glycolytic pathway, can also undergo direct oxidation to pentose phosphate which can be converted back into hexose monophosphate by a series of non-oxidative reactions. This series of reactions is now generally known as the pentose phosphate pathway. Considerable evidence has been documented in favour of the general occurrence of this pathway in a wide variety of biological tissues including plants (Axelrod & Beevers, 1956). Some of the key enzymes were purified from plant sources (Horecker, Smyrniotis & Klenow, 1953; Axelrod & Jang, 1954; Hurwitz, Weissbach, Horecker & Smyrniotis, 1956; Tabachnick, Srere, Cooper & Racker, 1958). Evidence in favour of the occurrence of this pathway *in vivo* mainly stems from the use of radioactive tracers and from the experiments with the inhibitors of the glycolytic pathway. The difficulties inherent in the use of isotopic techniques for this purpose are well recognized and the results obtained are often inconclusive (Utter, 1958). The isolation of the enzymes catalysing the reactions of the pentose phosphate pathway is another way of approach. Though considerable arguments have been made against this (Axelrod & Beevers, 1956), all other observations in favour of the operation of the pathway *in vivo* can be justified, provided that all the enzymes catalysing the reactions are found to occur in a single tissue.

In the present investigation germinating seedlings of mung bean (*Phaseolus radiatus*) have been chosen as the plant material to show the presence of all the enzymes involved in the direct oxidative pathway. Phosphoglucose dehydrogenase, the first enzyme in the sequence, has been partially purified.

MATERIALS AND METHODS

Seedlings of *Phaseolus radiatus* L. var. N.P. 28 were grown under standard conditions. The seeds were first sterilized by immersion in aq. 0·8% formaldehyde solution for 1 hr. and then thoroughly washed with sterile water and allowed to soak in the water under sterile conditions for 1 day. The seed coats were then removed with forceps, and the seeds were spread on a moist filter paper in a sterile Petri dish and kept at $25 \pm 1°$ in darkness. Seedlings were used for the experiments usually after 48 hr. of germination unless otherwise stated.

Dried baker's yeast used for the preparation of the phosphoglucose dehydrogenase was a gift from Dr Y. S. Lewis of the Central Food Technological Research Institute, Mysore, India.

Triphosphopyridine nucleotide (TPN), glucose 6-phosphate (G 6-P), diphosphopyridine nucleotide (DPN), reduced DPN (DPNH), 6-phosphogluconate (6-PG), fructose 6-phosphate (F 6-P), fructose diphosphate (FDP), ribose 5-phosphate (R 5-P), glutathione (GSH) and thiamine pyrophosphate were commercial products. Sedoheptulose 7-phosphate was a gift from Dr B. L. Horecker of the National Institutes of Health, Bethesda, Maryland, U.S.A. Xylulose 5-phosphate was prepared from R 5-P by the method of Hurwitz & Horecker (1956) with phosphoribose isomerase, which also contained phosphoribulose epimerase and a preparation of phosphoribulokinase.

Buffers. Phosphate buffers (0·1M) of various pH values were prepared by adding the requisite volume of 0·2M-NaH_2PO_4 (A) to the requisite volume of 0·2M-Na_2HPO_4 (B) and diluting the mixture to 200 ml.; pH 7·0, 39 ml. of A + 61 ml. of B; pH 7·2, 28 ml. of A + 72 ml. of B; pH 7·5, 16 ml. of A + 84 ml. of B.

Table 1. *Purification of phosphoglucose dehydrogenase from mung-bean seedling*

Details of the procedure are given in the text.

Steps	Activity: Units/ml.	Activity: Total units	Protein (mg./ml.)	Specific activity (unit/mg. of protein)
Crude extract	0·7	273	12·9	0·06
Ammonium sulphate I	8·0	160	26·9	0·30
Gel eluate and ammonium sulphate II	12·0	24	19·1	0·66

Veronal buffer (0·1 M), pH 7·5, was prepared by adding 30 ml. of 0·2 M-HCl to 50 ml. of 0·2 M-veronal and diluting the mixture to 200 ml.

2-Amino-2-hydroxymethylpropane-1:3-diol (tris) buffers, pH 7·0, 7·6 and 9·0, were prepared by adding the respective volumes of 47·0, 38·4 and 5·0 ml. of 0·2 M-HCl to 50 ml. of 0·2 M-tris and diluting each mixture to 200 ml.

The enzymes which were used in this investigation were isolated and purified in this Laboratory by methods as follows: phosphoglucose dehydrogenase was isolated and purified from baker's yeast following the method of Kornberg (1950). Phosphoglucose isomerase was isolated and purified from rabbit muscle after Slein (1950); the preparation was carried through the second ammonium sulphate fractionation and dialysed. The final diffusate was used for the assay. Aldolase was isolated and purified from rabbit muscle after the method of Taylor, Green & Cori (1948); the enzyme was used without recrystallization. Glycerophosphate dehydrogenase was isolated from rabbit muscle by the method of Racker (1947); the ammonium sulphate fraction which was precipitated between 50 and 70% saturation, was stored as a thick paste until needed. This fraction was contaminated with aldolase and triose phosphate isomerase, the presence of both of which was essential for a number of assays. Phosphoribulokinase was purified from *Spinacia oleracea* after the method of Hurwitz *et al.* (1956). The enzyme phosphoribose isomerase was isolated and purified from the acetone-dried powder of *Spinacia oleracea* (Chakravorty, 1958). This enzyme was carried through a number of steps, namely ammonium sulphate fractionation, heating at 60°, dialysis and a second ammonium sulphate fractionation. Sixtyfold purification was thus achieved.

Protein was determined by the Folin–Ciocalteu method (Lowry, Rosebrough, Farr & Randall, 1951). Phosphate measurements were made according to the method of Fiske & Subbarow (1925). Fructose was determined by the resorcinol method of Roe (1934). Assay for the pentulose was carried out by the cysteine carbazole colour reaction as described by Dische & Borenfreund (1951).

Purification of phosphoglucose dehydrogenase

The following operations were done at 0° unless otherwise stated. The mung-bean seedling (100 g.) after germination for 48 hr. was homogenized with 400 ml. of phosphate buffer (0·1 M), pH 7·2, in a Waring Blendor at top speed, first for 1 min. and then for 0·5 min. after allowing the warmed homogenate to cool. The homogenate was centrifuged at 30 000 *g* for 15 min. Volume of the supernatant was 390 ml. (crude extract). Ammonium sulphate (113 g.) was added and the precipitate was discarded. A further 49 g. of ammonium sulphate was added and the precipitate, collected by centrifuging, was dissolved in 15 ml. of water (ammonium sulphate I). The ammonium sulphate fraction (20 ml.) was treated with 100 ml. of freshly prepared calcium phosphate gel (12·4 mg./ml.). The gel collected by centrifuging was eluted twice, first with 100 ml. then with 50 ml. of 0·1 M-phosphate buffer, pH 7·5. Ammonium sulphate (34 g.) was added to 150 ml. of the eluate, the precipitate discarded and the precipitate formed by the addition of a further 18 g. of ammonium sulphate was collected by centrifuging and dissolved in 1·5 ml. of cold water. Final volume was 2 ml. (gel eluate and ammonium sulphate II). The activities at the different stages of purification are presented in Table 1. The enzyme has thus been purified 11-fold.

Preparation of the extract for the detection of other enzymes

Seedlings, after germination for 48 hr., were blended with 2 vol. of 0·1 M-phosphate buffer, pH 7·0, in a Waring Blendor for a total period of 1 min. (twice, each for 0·5 min.). The supernatant, collected by centrifuging, was used for the assay of the enzymes discussed below.

RESULTS

Phosphoglucose dehydrogenase and phosphogluconate dehydrogenase

These two enzymes mediate the first two oxidative steps in the pentose phosphate pathway. The presence of the two enzymes was detected by measuring the TPN reduction at 340 mμ with G 6-P and 6-PG as respective substrates:

$$(a)\quad \text{G 6-P} + \text{TPN}^+ \xrightarrow{\text{Phosphoglucose dehydrogenase}} \text{6-PG} + \text{TPNH} + \text{H}^+$$

$$(b)\quad \text{6-PG} + \text{TPN}^+ \xrightarrow{\text{Phosphogluconate dehydrogenase}} \text{R 5-P} + \text{CO}_2 + \text{TPNH} + \text{H}^+$$

The assay mixture contained 25 μmoles of veronal buffer, pH 7·5, 4 μmoles of G 6-P or 6-PG, 0·15 μmole of TPN and suitable amount of enzyme in a total volume of 3 ml. in a Beckman cuvette of 1 cm. light path. A control cuvette containing all

Table 2. *Phosphoglucose dehydrogenase and phosphogluconate dehydrogenase activities in the mung-bean seedling after different periods of germination*

Age of seedling (hr.)	Protein (mg./ml. of crude extract)	Phosphoglucose dehydrogenase activity		Phosphogluconate dehydrogenase activity	
		Unit/ml. of crude extract	Specific activity (unit/mg. of protein)	Unit/ml. of crude extract	Specific activity (unit/mg. of protein)
24	9·9	0·50	0·05	0·50	0·05
48	7·7	0·50	0·06	0·40	0·05
72	4·8	0·27	0·05	0·25	0·05
96	3·2	0·08	0·02	0·11	0·03

the above components except the substrate (G 6-P or 6-PG) was simultaneously run. The increase in extinction at 340 mμ was followed after the addition of the enzyme. Extinction readings were corrected for the control. The increase in extinction with G 6-P as substrate measures both phosphoglucose dehydrogenase and phosphogluconate dehydrogenase activities. The difference in the rates of increase of the extinctions with G 6-P and 6-PG as substrates denotes the phosphogluconate dehydrogenase activity. One unit of enzyme is defined as that amount which causes an initial change in extinction of 1·0 per min. under the above-mentioned conditions (Kornberg, 1950).

Variation of activities of dehydrogenases with age of seedling

Some preliminary experiments were done to determine the activities of the dehydrogenases in the seedling after different periods of germination. At requisite intervals 5 g. batches of seedlings were treated as described in the Methods section for the preparation of the crude extract. This was assayed for phosphoglucose and phosphogluconate dehydrogenase activities. The results presented in Table 2 show that the activities decline considerably after germination for 72 hr. Seedlings after germination for 48 hr. were therefore invariably used for the preparation of phosphoglucose dehydrogenase.

Properties of phosphoglucose dehydrogenase

The partially purified preparation showed little phosphogluconate dehydrogenase activity and was active only with TPN and showed no activity with DPN (Table 3). The enzyme shows no Mg^{2+} ion requirement in veronal (Table 3), which was always used in the assay system. There was practically no inhibition of the enzyme activity by phosphate up to a concentration of 0·1 M, but in concentrations of 0·2 and 0·9 M about 28 and 97 % inhibition was observed. The enzyme showed maximum activity at pH 7·5 and little activity below pH 6·0 and above pH 9·0. The properties of the dehydrogenase are thus similar to those of the phosphoglucose dehydrogenase from yeast (Kornberg, 1950).

Table 3. *Effect of* Mg^{2+} *ion, diphosphopyridine nucleotide and phosphate on phosphoglucose dehydrogenase from mung-bean seedling*

The assay system is described in the text except that veronal buffer was omitted when phosphate was tested.

Additions	Concentration (M)	Activity	
		Units/ml. of crude extract	Percentage of control
None	—	6·0	100
Mg^{2+}	$0{\cdot}66 \times 10^{-2}$	5·8	97
Mg^{2+}	$6{\cdot}60 \times 10^{-2}$	6·0	100
DPN*	$5{\cdot}0 \times 10^{-5}$	0·0	0
DPN	$5{\cdot}0 \times 10^{-5}$	5·9	99
Phosphate	0·1	5·8	97
Phosphate	0·2	4·3	72
Phosphate	0·9	0·2	3

* TPN was omitted from the assay system in this case.

Table 4. *Phosphoribose isomerase activity in extracts of mung-bean seedling*

For test conditions see text.

Protein added (mg.)	Increase in $E_{540\,m\mu}$ (corrected for the blank)	Activity (unit/mg. of protein)
0·50	0·195	0·39
1·00	0·408	0·41

Phosphoribose isomerase

The occurrence of phosphoribose isomerase (PRI) in the extract was demonstrated by the formation of ribulose 5-phosphate (Ru 5-P) from R 5-P by the standard cysteine carbazole colour reaction of the pentulose (Hurwitz *et al.* 1956).

$$\text{R 5-P} \xrightleftharpoons{\text{PRI}} \text{Ru 5-P}$$

The incubation mixture contained 30 μmoles of tris buffer, pH 7·0, 1 μmole of cysteine and 2 μmoles of R 5-P, in a total volume of 0·8 ml. The incubation was carried out at 30° for 10 min. and the reaction was stopped by the addition of 2·5 ml. of aq. 70 % H_2SO_4. Immediately 0·1 ml. of 1·2 % solution of cysteine hydrochloride (freshly prepared) was added, thoroughly mixed and then

0·1 ml. of ethanolic 0·1 % carbazole solution was added. Absorption readings at 540 mμ were taken after 10 min. The results of the assay are presented in Table 4. One unit is defined as that which produces an absorption of 1·0 under the assay conditions.

Phosphoribulose epimerase (3-epimerase)

The presence of 3-epimerase in the germinating mung bean was demonstrated by the assay method as described by Hurwitz & Horecker (1956)

Xylulose 5-phosphate
⇅ Epimerase
Ru 5-P ⇌ (Isomerase) R 5-P

The measurement was based on the relative stability of R 5-P to alkali. The incubation was carried out for 5 min. at 35° in a total volume of 0·2 ml. containing 10 μmoles of tris buffer, pH 7·5, 14 μg. of phosphoribose isomerase, suitable amount of enzyme and 0·13 μmole of xylulose 5-phosphate. The reaction was stopped by adding 0·2 ml. of 2M-KOH and the mixture was kept at 35° for 20 min. Then the orcinol-reactive material was measured by the method of Mejbaum (1939) with 40 min. heating period. A blank was run without addition of enzyme and suitable corrections were accordingly made. The results are presented in Table 5.

Table 5. *Phosphoribulose epimerase activity in extracts of mung-bean seedling*

Details of the assay system are given in the text.

Protein added (μg.)	$E_{670\ m\mu}$ (corrected for the blank)	Alkali-stable pentose phosphate formed (μmole)
90	0·495	0·032
180	0·915	0·059

Transketolase

Transketolase activity of the extract was shown by measuring the formation of glyceraldehyde 3-phosphate from R 5-P and xylulose 5-phosphate as described by de La Haba, Leder & Racker (1954). Xylulose 5-phosphate was generated from R 5-P by the addition of phosphoribose isomerase (PRI) containing 3-epimerase. Glyceraldehyde 3-phosphate formed was measured by DPNH oxidation subsequent to the conversion of the former into α-glycerophosphate by the combined action of triose phosphate isomerase and α-glycerophosphate dehydrogenase.

(*a*) R 5-P $\overset{PRI}{\rightleftharpoons}$ Ru 5-P

(*b*) Ru 5-P $\overset{3\text{-epimerase}}{\rightleftharpoons}$ xylulose 5-phosphate

(*c*) Xylulose 5-phosphate + R 5-P $\overset{Transketolase}{\rightleftharpoons}$ glyceraldehyde 3-phosphate + sedoheptulose 7-phosphate

(*d*) Glyceraldehyde 3-phosphate $\overset{Triose\ phosphate\ isomerase}{\rightleftharpoons}$ dihydroxyacetone phosphate

(*e*) Dihydroxyacetone phosphate + DPNH + H^+ $\xrightarrow{Glycerophosphate\ dehydrogenase}$ α-glycerophosphate + DPN^+

Table 6. *Transketolase activity in extracts of mung-bean seedling*

Details of the test system are given in the text.

Protein added (mg.)	$E_{340\ m\mu}$/3 min.	Triose phosphate formed/3 min. (μmole)
None	0·005	0·001
0·25	0·130	0·031
0·50	0·250	0·060

The assay system contained, in a total volume of 1·5 ml., 25 μmoles of tris buffer, pH 7·6, 0·5 μmole of thiamine pyrophosphate, 1 μmole of $MgCl_2$, 2 μmoles of R 5-P and 70 μg. of phosphoribose isomerase. Incubation was at room temperature for 6 min., and then 0·1 mg. of α-glycerophosphate dehydrogenase (contaminated with triose phosphate isomerase) and a suitable amount of DPNH, to give an absorption reading of 0·5–0·6 at 340 mμ, were added. Before adding the extract containing transketolase to start the reaction the extinction at 340 mμ was observed for a while to check whether there was any oxidation of DPNH owing to the contamination of phosphoribose isomerase or α-glycerophosphate dehydrogenase or both with transketolase activity. They were, however, found to be free of transketolase. The results obtained with the seedling extract are presented in Table 6.

Transaldolase

The transaldolase activity in the extract was detected by measuring the rate of formation of F 6-P from sedoheptulose 7-phosphate and glycer-

Table 7. *Transaldolase activity in extracts of mung-bean seedling*

For test conditions see text.

Protein added (mg.)	Substance omitted	$\Delta E_{340\,m\mu}$/4·5 min.	$E_{340\,m\mu}$ (corrected for the blank)	TPN reduced (μmole)
None	—	0·017	—	—
0·65	—	0·070	0·053	0·013
1·30	—	0·131	0·114	0·028
0·65	Sedoheptulose 7-phosphate	0·042	0·025	0·006

Table 8. *Phosphoglucose isomerase activity in extracts of mung-bean seedling*

For test conditions see text.

Protein added (μg.)	F 6-P formed (μmole/10 min.)	Activity (units/mg. of protein)
26	0·12	4·6
52	0·20	3·8

aldehyde 3-phosphate (Horecker & Smyrniotis, 1953). Glyceraldehyde 3-phosphate was generated from FDP by muscle aldolase. F 6-P was assayed by the reduction of TPN in the presence of phosphoglucose isomerase (PGI) and phosphoglucose dehydrogenase.

(*a*) FDP $\overset{\text{Aldolase}}{\rightleftharpoons}$ glyceraldehyde 3-phosphate

(*b*) Sedoheptulose 7-phosphate + glyceraldehyde 3-phosphate $\overset{\text{Transaldolase}}{\rightleftharpoons}$ F 6-P + erythrose 4-phosphate

(*c*) F 6-P $\overset{\text{PGI}}{\rightleftharpoons}$ G 6-P

(*d*) G 6-P + TPN^+ $\overset{\text{Phosphoglucose dehydrogenase}}{\rightleftharpoons}$ 6-PG + TPNH + H^+

The assay system contained, in a final volume of 15 ml., 50 μmoles of tris buffer, pH 7·0, 0·15 μmole of TPN, 0·5 μmole of FDP, 0·4 μmole of sedoheptulose 7-phosphate, 14 μg. of aldolase, 7·4 μg. of phosphoglucose isomerase and 7 μg. of phosphoglucose dehydrogenase. The assay system was incubated without the seedling extract at room temperature for 5 min., in a Beckman 1 cm. cuvette, and then the extract to be tested was added. A control incubation was run excluding the crude extract as the system showed slight TPN reduction even without addition of the extract. The absorption readings were corrected for the control. Another control incubation was run, with sedoheptulose 7-phosphate omitted, to show that the F 6-P was not formed solely by the action of phosphatase, if any, on FDP. The results are presented in Table 7.

Phosphoglucose isomerase

Phosphoglucose isomerase (PGI) was determined by measuring the formation of F 6-P from G 6-P. F 6-P was assayed by the resorcinol method of Roe (1934).

$$\text{G 6-P} \overset{\text{PGI}}{\rightleftharpoons} \text{F 6-P}$$

The incubation mixture contained 47 μmoles of tris buffer, pH 9·0, 1 μmole of G 6-P and the extract to be tested, in a final volume of 1 ml. The incubation was carried out at 30° for 10 min. The reaction was stopped by adding 3·5 ml. of 10 M-HCl. Then 0·5 ml. of resorcinol reagent was added, mixed well and heated for 10 min. in a bath, at 80°, followed by cooling in water for 5 min. The absorbancy readings were taken at 510 mμ. Suitable blank and a standard F 6-P solution were run simultaneously. The results are presented in Table 8. One unit of enzyme is defined as that which produces 1 μmole of F 6-P under the standard assay conditions.

DISCUSSION

The evidence presented above shows the presence of all the enzymes of the pentose phosphate pathway in extracts of germinating mung-bean seedling. Though the presence of these enzymes does not necessarily prove that the pentose phosphate pathway as a whole is operative *in vivo*, it is not unreasonable to suggest that this pathway is of significance in the germinating seedling. The activity of both phosphoglucose dehydrogenase and phosphogluconate dehydrogenase, which catalyse the initial steps of the pentose phosphate pathway, remain more or less constant up to the age of 48 hr., but after this there is a fall. Germination for longer periods than 96 hr. results in the virtual disappearance of both the dehydrogenases from the extract. Though so far there is no evidence in

favour of energy production by the pentose phosphate pathway, the occurrence of this oxidative cycle in the early life of the seedling emphasizes its close association with the high metabolic activity at this stage.

The activities of the different enzymes in the extract, as judged from the number of units per ml. and from the activity in relation to the protein content, were low in comparison with reported activities of the enzymes in extracts from animal and bacterial systems. This is, however, to be expected because of the presence of considerable amounts of non-enzymic protein (storage protein) in the seeds and in the seedling at the early stages of germination.

Phosphoglucose dehydrogenase which has been partially purified has the pH optimum and other properties similar to those of the yeast enzyme. Unlike bacterial enzyme it is specific for TPN and inactive with DPN.

SUMMARY

1. The occurrence in extracts of germinating mung-bean seedling of the following enzymes of the oxidative pentose phosphate pathway has been shown: phosphoglucose dehydrogenase, phosphogluconate dehydrogenase, phosphoribose isomerase, phosphoribulose epimerase, transketolase, transaldolase and phosphoglucose isomerase.

2. During the initial period of germination (up to 48 hr.) the amounts and specific activities of the dehydrogenases increase with time. After 72 hr., however, both start decreasing and after 96 hr. the enzymes disappear from the extracts.

3. The enzyme phosphoglucose dehydrogenase has been partially purified and its properties have been found to be similar to those of the yeast enzyme. The enzyme is triphosphopyridine nucleotide-specific, and has been successfully used in the assay of glucose 6-phosphate.

REFERENCES

Axelrod, B. & Beevers, H. (1956). *Annu. Rev. Pl. Physiol.* **7**, 267.
Axelrod, B. & Jang, R. (1954). *J. biol. Chem.* **209**, 847.
Chakravorty, M. (1958). *Sci. & Cult.* **24**, 380.
de La Haba, G., Leder, I. G. & Racker, E. (1954). *J. biol. Chem.* **214**, 409.
Dische, Z. & Borenfreund, E. (1951). *J. biol. Chem.* **192**, 583.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Horecker, B. L. & Smyrniotis, P. Z. (1953). *J. Amer. chem. Soc.* **75**, 2021.
Horecker, B. L., Smyrniotis, P. Z. & Klenow, H. (1953). *J. biol. Chem.* **205**, 661.
Hurwitz, J. & Horecker, B. L. (1956). *J. biol. Chem.* **223**, 993.
Hurwitz, J., Weissbach, A., Horecker, B. L. & Smyrniotis, P. Z. (1956). *J. biol. Chem.* **218**, 769.
Kornberg, A. (1950). *J. biol. Chem.* **182**, 805.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Mejbaum, W. Z. (1939). *Hoppe-Seyl. Z.* **258**, 117.
Racker, E. (1947). *J. biol. Chem.* **167**, 843.
Roe, J. H. (1934). *J. biol. Chem.* **107**, 15.
Slein, M. W. (1950). *J. biol. Chem.* **186**, 753.
Tabachnick, M., Srere, P. A., Cooper, J. & Racker, E. (1958). *Arch. Biochem. Biophys.* **74**, 315.
Taylor, J. F., Green, A. & Cori, G. T. (1948). *J. biol. Chem.* **173**, 591.
Utter, M. F. (1958). *Annu. Rev. Biochem.* **27**, 245.

The Metabolism of Exogenous ^{14}C-Labelled 5-Hydroxytryptamine in Rats

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In spite of intensive study of 5-hydroxytryptamine from both biochemical and biological aspects knowledge of its metabolism is still incomplete. The fate and distribution of 5-hydroxytryptamine in rats was studied by Erspamer (1955, 1956), but more specific information about an overall metabolism is still lacking. 5-Hydroxyindolylacetic acid was established as a metabolite by Udenfriend, Titus & Weissbach (1955) and Erspamer (1955). Weissbach, Redfield & Udenfriend (1957) found 5-hydroxyindolylacetaldehyde in homogenates of guinea-pig liver. Curzon (1957) found 5-hydroxyindolylacetic acid *O*-sulphate in urine of argentaffinoma subjects and in urine of rats after administration of 5-hydroxytryptophan, 5-hydroxytryptamine and 5-hydroxyindolylacetic acid. Chadwick & Wilkinson (1958) established 5-hydroxytryptamine *O*-sulphate as a metabolite of 5-hydroxytryptamine in incubated rat-liver homogenates. Weissbach, Redfield & Udenfriend (1958) identified 5-hydroxytryptamine *O*-glucuronide as a normal metabolite of 5-hydroxytryptamine in the urine of

mice. The amount of this compound greatly increased when mice had been pre-treated with *iso*propyl*iso*nicotinic acid hydrazide (iproniazid). McIsaac & Page (1958) investigated carcinoid human urine and identified, by paper chromatography, oxytocic activity and fluorescent spectra, besides 5-hydroxyindolylacetic acid and indican two new metabolites of 5-hydroxytryptamine: 1′-*N*-acetyl-5-hydroxytryptamine and 5-hydroxyindolylaceturic acid. They also claimed to have found in radioautographs obtained from urinary extracts of rats and rabbits given radioactive 5-hydroxytryptamine the same metabolites, with the addition of two other minor metabolites. One of these has been identified as 5-hydroxytryptamine *O*-glucuronide. However, most of the papers about new metabolites of 5-hydroxytryptamine are preliminary reports in which only few experimental details are given.

In the present work an attempt was made, by using 5-hydroxy[^{14}C]tryptamine labelled at the end of the side chain (Keglević-Brovet, Kveder & Iskrić, 1957), to obtain some information about the overall metabolism of exogenous 5-hydroxytryptamine in rats, to establish the number of the main metabolites of 5-hydroxytryptamine excreted in urine and to determine chromatographic characteristics of these. A preliminary account of some of the results has been given by Keglević-Brovet, Supek, Kveder, Iskrić & Kečkeš (1958).

METHODS

5-Hydroxy[1′-^{14}C]tryptamine, as the creatinine sulphate complex (Keglević-Brovet *et al.* 1957), with a specific activity of 0·69 mc/m-mole, was used in all experiments.

Animals and dosage. Adult male albino rats from our Institute stock (aged 3–4 months), maintained on a standard diet (Coward, 1947), were employed throughout. In all experiments the dose level was 2·9 mg./100 g. body wt. for 5-hydroxy[1′-^{14}C]tryptamine creatinine sulphate (1·27 mg. of 5-hydroxytryptamine/100 g. body wt.). The compound was injected subcutaneously, dissolved in 1 ml. of 0·9 % NaCl, and the syringe was rinsed twice with 1 ml. of the same solution, a metal three-way stopcock being used. During the experiment the animals were allowed water but not food.

Experiments 1–4. These are the experiments described by Keglević-Brovet *et al.* (1958).

Experiments 3–15. Each animal was kept in a metabolism cage designed for the separate collection of urine and faeces. Expts. 3–4, 10–12 and 13–15 were run simultaneously.

Collection of urine. In all experiments urine was collected separately for the periods 0–12 and 12–14 hr. in tubes cooled by ice. In Expts. 1–4 the whole urine was freeze-dried. In Expts. 5–6 urine as collected was investigated and only a small fraction was freeze-dried. In Expts. 7–15 all the urine samples were made up to 20 ml. and pipetted in 1 ml. portions into tared 2 ml. vials. About 15 vials of each urine sample were freeze-dried, sealed and reweighed. The weights of the dry residues agreed within ±2·5 %. To keep identical drying conditions in Expts. 10–12 and 13–15 the urine vials were freeze-dried synchronously on the same pump.

Estimation of ^{14}C *in samples.* Freeze-dried urine, faeces and tissues as well as untreated urine were subjected to wet combustion by the Van Slyke & Folch (1940) reagent in the apparatus by Weisburger, Weisburger & Morris (1952); the resulting CO_2 was absorbed in 1 % (w/v) carbonate-free NaOH solution and converted into $BaCO_3$ by standard procedure (Calvin, Heidelberger, Reid, Tolberg & Yankwich, 1949). In Expts. 7–15 the vial containing dry residue corresponding to 1 ml. of the whole urine volume was cut in the middle and both parts were dropped into the combustion tube.

Determination of radioactivity. Samples of $Ba^{14}CO_3$ (2·83 cm.^{2}) were counted at infinite thickness on standard plates (Tracerlab Inc.; E-7 B). The counts were corrected when necessary for dead time, and the count rates of samples less than infinitely thick to the values of infinite thickness. Sufficient counts were recorded to give a standard error of ±5 % for the lowest activities and ±3 % for higher activities. $Ba^{14}CO_3$ standards were prepared from the 5-hydroxytryptamine preparation used in these experiments.

Paper chromatography. Portions of freeze-dried urine were dissolved in water and subjected to descending chromatography on strips (54 cm. × 6 cm.) of Whatman no. 1 filter paper. The sample was applied to the paper as a streak about 5 cm. long. The four solvent systems used are listed in Fig. 2. Two-dimensional chromatograms were run in all combinations of the four solvent systems. In order to eliminate interfering substances some specimens of urine were treated with deactivated charcoal according to the procedure of Dalgliesh (1955). The phenolic eluates were evaporated *in vacuo* and the residue was subjected to one- and two-dimensional paper chromatography.

Detection of radioactive spots. Radioautographs were obtained from chromatograms kept in contact with Ferrania X-ray film for 5–10 days. One-dimensional chromatograms were scanned with an automatic scanner, consisting of a Geiger–Müller mica-window counter attached to an automatic recorder.

Elution of radioactive spots. Radioactive areas of the one-dimensional chromatograms were cut out and eluted with water. The transfer was performed as a continuous process, the eluates being dried simultaneously by a jet of air directed against the under side of the new paper strip (Moore & Boylen, 1953). The eluates were then rechromatographed separately in the three remaining solvent systems. Chromatograms obtained in this way were scanned, radioactive areas were re-eluted and individually subjected to chromatography in the original solvent system.

Tests for uronic acid. The eluates were checked by the carbazole method of Dische (1947) and the naphtharesorcinol test of Bray, Humphris, Thorpe, White & Wood (1952).

Bioassay. Oxytocic activity was tested on the isolated atropinized uterus of the rat in oestrus (Amin, Crawford & Gaddum, 1954).

SYNTHESES

All melting points are uncorrected.

5-*Benzyloxyindolylaceturic acid ethyl ester* (I). 5-Benzyloxyindolylacetic acid (Stoll, Troxler, Peyer & Hofmann, 1955) (1·4 g., 5 m-moles) and *N*-carbonylglycine ethyl

ester, prepared according to Goldschmidt & Wick (1952) (0·7 g., 5·5 m-moles), were dissolved in toluene (10 ml.), warmed to 80° until the evolution of CO_2 ceased (about 1 hr.) and then refluxed at 110° for a further 1 hr. Toluene was removed *in vacuo*, the reddish gummy residue was dissolved in hot ethanol and filtered and kept at 0° overnight. Brown crystals (0·9 g.) separated. They were dissolved in ethyl acetate, treated with charcoal and precipitated by light petroleum (b.p. 60–80°). Two further recrystallizations from methanol–water gave white crystals (0·7 g., 38%) of m.p. 112–114° (Found: C, 69·1; H, 6·1; N, 7·8. $C_{21}H_{22}O_4N_2$ requires C, 68·8; H, 6·1; N, 7·7%).

5-Benzyloxyindolylaceturic acid (II). The ester (I) (366 mg., 1 m-mole) was moistened with ethanol, 0·5 N-NaOH (2·5 ml.) was added and the mixture heated to 70° on a water bath with occasional shaking until it dissolved completely. It was then cooled and kept at room temperature for 3 hr. After acidification with acetic acid, small crystals separated. They were filtered off, washed with aq. 1% ethanol and dried (318 mg.). Crystallization from hot ethanol with addition of warm water to cause turbidity gave white crystals (268 mg., 79%) of m.p. 143–145° (Found: C, 67·6; H, 5·5. $C_{19}H_{18}O_4N_2$ requires C, 67·4; H, 5·4%).

5-Hydroxyindolylaceturic acid (III). Compound (II) (280 mg., 1·16 m-moles), dissolved in methanol (10 ml.), was catalytically debenzylated at s.t.p., with 10% Pd–$BaSO_4$ (100 mg.). After 5 hr. the reduction was complete and the catalyst was removed by centrifuging. Methanol was completely removed by a stream of N_2, the residue dissolved in acetone (3 ml.) and the solution was filtered. To the clear solution $CHCl_3$ was added until a slight turbidity persisted, and kept at 0° for 3 days with slight further addition of $CHCl_3$. White, long prisms (166 mg., 80%), m.p. 183–185°, were obtained. After two recrystallizations the m.p. was 186–187° (134 mg., 66%) (Found: C, 58·0; H, 5·0; N, 11·4. $C_{12}H_{12}O_4N_2$ requires C, 58·1; H, 4·9; N, 11·3%). The infrared-absorption spectrum of this substance is shown in Fig. 1.

Hydrolysis of 5-hydroxyindolylaceturic acid. Compound (III) (5 mg.), dissolved in 3 N-HCl (2 ml.), was heated on a boiling-water bath for 0·5 hr. The solution was evaporated to dryness *in vacuo*, the residue was dissolved in water and the solution again evaporated to remove the traces of HCl. The dark residue was dissolved in water and submitted to paper chromatography in four solvent systems.

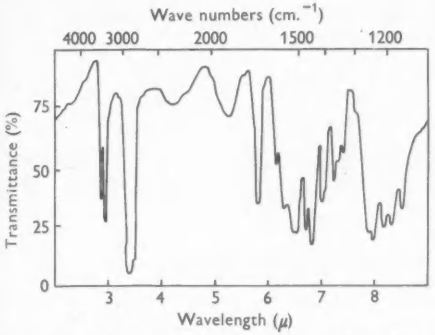

Fig. 1. Infrared-absorption spectrum of 5-hydroxyindolylaceturic acid in Nujol (liquid paraffin). A Perkin–Elmer double-beam automatic recording spectrophotometer (NaCl prisms) was used.

RESULTS

Table 1 shows radioactivity recovered in urines from 15 rats. The volume of urine passed by each rat during the two periods of collection has no connexion with either the weight of total solids or the radioactivity recovered. The great differences in the recoveries of radioactivity in 0–12 hr. urines cannot be satisfactorily explained. Urine collected in the 12–24 hr. period showed a much lower radioactivity in all experiments and there is no evidence of a higher recovery to compensate for a low recovery in 0–12 hr. urines.

Paper chromatograms of urine from all experiments were scanned for radioactivity and always showed the same qualitative pattern. However, the quantitative relation of radioactivity between single metabolites differed slightly in individual experiments. Fig. 2 shows the typical diagrams of 0–12 hr. urine run in four solvent systems. Several radioactive compounds are excreted. The R_F values of individual radioactive compounds were determined by elution and rechromatography (Table 2). Only the compounds which retained after the whole procedure the same R_F in the original solvent system were accepted as metabolites. Other radioactive spots with no reproducible chromatographic behaviour were ascribed to degradation products.

The identification of 5-hydroxyindolylacetic acid and unchanged 5-hydroxytryptamine was established by comparing their chromatographic behaviour with pure synthetic substances. In addition, 5-hydroxytryptamine was checked by testing the eluates for oxytocic activity. In solvent system 4 the R_F of 5-hydroxytryptamine found in urine did not correspond with the R_F (0·33) of synthetic 5-hydroxytryptamine creatinine sulphate. Besides 5-hydroxyindolylacetic acid five other radioactive metabolites have been detected.

To judge from the radioactive areas of scanner diagrams, 5-hydroxyindolylacetic acid accounted for 50–60% of radioactivity applied to the chromatograms. Compounds A, B and C contained about 25–30% of the total radioactivity, C always being the most active among them. D, E and unchanged 5-hydroxytryptamine accounted for 10–15% of the radioactivity, of which the smallest part was due to 5-hydroxytryptamine. Compound F could not be taken decisively as a metabolite of 5-hydroxytryptamine, because in elution and rechromatography its presence was confirmed with

Table 1. *Recovery of radioactivity in rat urine after injection of 5-hydroxy[1′-^{14}C]tryptamine*

The injected dose of 5-hydroxy[1′-^{14}C]tryptamine (in the form of creatinine sulphate complex) was 2·9 mg./100 g. body wt. (1·27 mg. of the free base/100 g. body wt.) with a specific activity of 0·69 mc/m-mole. Standard $BaCO_3$ samples were prepared from the same preparation. In all experiments except Expts. 5–6 urine was freeze-dried. All measurements of radioactivity were on $BaCO_3$ mounted at infinite thickness on 2·83 cm.^{2} plates. In Expt. 1 radioactive measurements were made with a Geiger–Müller mica-window tube and in Expts. 2–15 with a windowless flow counter (Tracerlab Inc., SC-16). Expts. 1–4 are from Keglević-Brovet, Supek, Kveder, Iskrić & Kočkeš (1958). Mean values ± S.E.M. are given with ranges in parentheses; superior figures indicate the number of determinations.

Expt. no.	10^{-5} × Total radioactivity injected (counts/min.)	Urine excreted (ml.)		10^{-5} × Total radioactivity of urine (counts/min.)		Percentage of injected radioactivity recovered in urine	
		0–12 hr.	12–24 hr.	0–12 hr.	12–24 hr.	0–12 hr.	12–24 hr.
1	8·05	10·0	9·5	2·92 (2·79–3·05)^4	0·15 (0·14–0·16)^4	36·3 ± 0·70 (34·7–37·9)^4	1·9 ± 0·05 (1·8–2·0)^4
2	24·46	5·6	9·5	12·38 (11·31–13·54)^4	0·42 (0·36–0·51)^4	50·6 ± 1·92 (46·2–55·4)^4	1·7 ± 0·13 (1·5–2·1)^4
3	21·13	7·5	12·0	8·77 (8·31–9·31)^4	0·40 (0·34–0·45)^4	41·5 ± 1·07 (39·3–44·1)^4	1·9 ± 0·12 (1·6–2·1)^4
4	26·25	9·3	11·4	11·07 (10·92–11·26)^4	0·27 (0·24–0·29)^4	42·2 ± 0·29 (41·6–42·9)^4	1·0 ± 0·05 (0·9–1·1)^4
5	24·96	10·5	11·5	14·39 (12·00–18·56)^{12}	1·99 (1·91–2·04)^3	57·7 ± 2·44 (47·9–74·4)^{12}	7·9 ± 0·17 (7·6–8·2)^3
6	24·25	19·0	9·0	17·35 (16·50–18·84) ^{8}	0·47 (0·46–0·49)^3	71·5 ± 1·07 (68·0–77·7)^8	1·9 ± 0·04 (1·9–2·0)^3
7	31·36	7·6	12·8	22·73 (21·52–24·35)^4	1·67 (1·60–1·72)^3	72·5 ± 1·94 (68·6–77·7)^4	5·3 ± 0·12 (5·1–5·5)^3
8	26·46	2·6	4·4	18·67 (17·69–19·53)^6	1·64 (1·52–1·72)^6	70·6 ± 1·02 (66·9–73·8)^6	6·2 ± 0·10 (5·8–6·5)^6
9	25·71	11·0	8·0	13·24 (13·13–13·34)^5	0·79 (0·73–0·84)^2	51·6 ± 0·19 (51·0–51·9)^5	3·1 ± 0·25 (2·8–3·3)^2
10	22·43	7·4	12·5	18·07 (17·27–18·69)^6	1·27 (1·24–1·29)^2	80·6 ± 0·87 (77·0–83·3)^6	5·7 ± 0·15 (5·5–5·8)^2
11	21·91	9·0	7·0	16·93 (16·31–17·39)^4	1·31 (1·29–1·35)^3	77·3 ± 1·17 (74·4–79·4)^4	6·0 ± 0·10 (5·9–6·2)^3
12	23·24	4·8	11·5	15·28 (14·23–16·12)^4	1·94 (1·88–1·99)^3	65·8 ± 2·09 (61·3–69·4)^4	8·3 ± 0·15 (8·1–8·6)^3
13	23·30	13·4	7·0	9·86 (9·12–10·62)^7	1·94 (1·88–2·00)^3	42·3 ± 0·93 (39·2–45·6)^7	8·2 ± 0·07 (8·1–8·3)^3
14	23·78	11·4	13·0	19·70 (18·54–20·60)^4	0·63 (0·62–0·65)^3	82·8 ± 1·95 (78·0–86·6)^4	2·7 ± 0·07 (2·6–2·8)^3
15	24·09	13·6	19·0	14·56 (13·92–15·10)^4	0·90 (0·83–0·94)^3	60·4 ± 1·00 (57·8–62·7)^4	3·8 ± 0·18 (3·5–3·9)^3

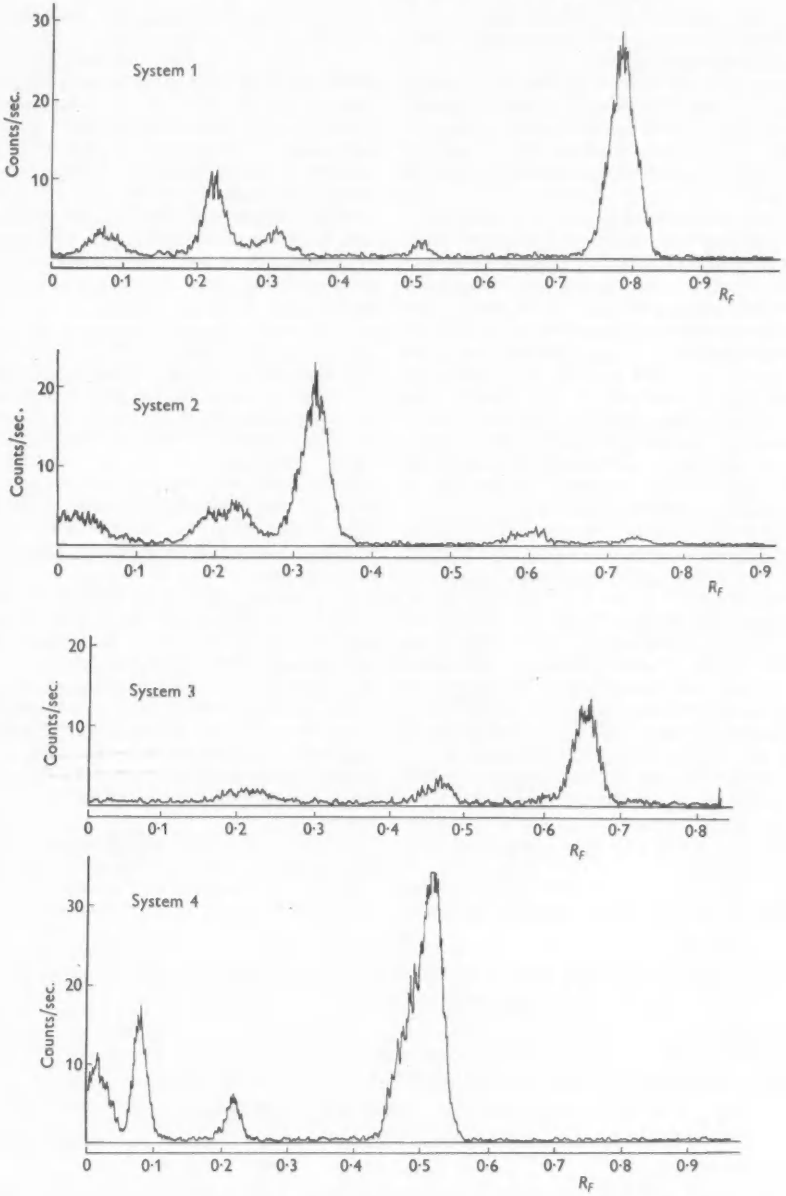

Fig. 2. Typical continuous scanning records of radioactive spots on chromatograms of 12 hr. urine of rats injected with 5-hydroxy[1′-^{14}C]tryptamine. The descending method on Whatman no. 1 paper was used. Solvent systems (by vol.) and times of run: 1, butanol–acetic acid–water (4:1:5; 19 hr.); 2, propan-2-ol–aq. NH_3 soln.–water (10:1:1; 22 hr.); 3, methanol–benzene–butanol–water (4:2:2:2; 14 hr.); 4, butanol–ethanol–water (4:1:1; 20 hr.).

certainty only in solvent systems 1 and 2. Its radioactivity amounted to not more than 2% of the total urinary activity.

All the compounds located gave a positive test with Ehrlich's reagent. Exposure of chromatograms to ultraviolet light revealed no fluorescence of radioactive spots. Compounds A and D gave a positive naphtharesorcinol and carbazole test for glucuronides.

Radioautographs obtained from two-dimensional paper chromatograms confirmed the location of metabolites found by one-dimensional technique. Combination of solvent system 2 followed by system 1 was the most efficient. Good results were also obtained with combination of system 4 followed by system 3. Other combinations of the four solvent systems gave a rather poor resolution.

A much better resolution in all systems was achieved by submitting urine to deactivated charcoal treatment (after Dalgliesh, 1955). The relative amounts of radioactive compounds changed after this treatment because of different absorption on the charcoal. 5-Hydroxytryptamine vanished completely and could not be detected either by scanning analysis or radioautography. The amount of 5-hydroxyindolylacetic acid was decidedly lower. Radioactive peaks of one-dimensional chromatograms in system 3 resolved after this procedure, giving compounds A, B, C and D as individual peaks. Two-dimensional radioautographs of charcoal-treated urine are shown in Fig. 3. All metabolites, including the compound F, could be detected. Moreover, two additional weak spots, D_1 and D_2, appeared. As they could not be detected by elution and rechromatography under milder conditions than charcoal treatment, they are believed to be degradation products. D_1 is the only radioactive spot not giving a positive test with Ehrlich's reagent.

Scanner diagrams and two-dimensional radioautographs of 12–24 hr. urine revealed the same pattern as in 0–12 hr. urine, but of very low intensity.

R_F values of 5-hydroxyindolylaceturic acid and of the products obtained after acid hydrolysis are summarized in Table 3. The R_F values of 5-hydroxyindolylaceturic acid differ considerably from those of any radioactive metabolite found in rat urine. The substance was also added to the freeze-dried residue of 0–12 hr. urine and the mixture chromatographed in the usual manner. The R_F values of this compound were not changed by this procedure, and the spots were distinct from the radioactive spots in all four solvent systems used.

DISCUSSION

The dose of injected 5-hydroxytryptamine in the experiments described was rather high. The rats showed pronounced toxic effects in the first 6 hr. but then seemed to recover completely. Histological examinations revealed only mild renal injuries typical of 5-hydroxytryptamine. Therefore it seems that these experiments, in spite of the high dose, afford an insight into the normal metabolic pathways of exogenous 5-hydroxytryptamine in rats.

The results obtained in Expts. 1–4 (Keglević-Brovet *et al.* 1958) have shown that about 2% of administered radioactivity was expired, the most rapid elimination of $^{14}CO_2$ occurring 8–14 hr. after the injection. The low percentage of radioactivity found in faeces (about 2%) and retained in the body (about 6%), as well as in all 12–24 hr. urines, supports the presumption that the most important pathway of metabolism of 5-hydroxytryptamine is the excretion in urine during the first 12 hr.

However, in some experiments radioactivity amounting up to 40% of the total dose remained undetected. The great differences in radioactive recoveries obtained with freeze-dried 12 hr. urine

Table 2. *R_F values and colour reaction of the main radioactive compounds in rat urine after injection of 5-hydroxy[1′-^{14}C]tryptamine*

Each radioactive spot of the original chromatograms of 12 hr. urine in solvent systems quoted in Fig. 2 was eluted and rechromatographed in the three remaining solvent systems; the separated radioactive spots were re-eluted and individually subjected to chromatography in the original solvent system. A–F are metabolites.

	R_F in solvent system				Intensity of colour with Ehrlich's reagent
	1	2	3	4	
5-Hydroxytryptamine	0·50	0·61	0·68	0·52	(+)
5-Hydroxyindolylacetic acid	0·78	0·32	0·68	0·52	+ + +
A	0·09	0·03	0·22	0·02	+ +
B	0·23	0·03	0·22	0·02	+ +
C	0·23	0·21	0·46	0·07	+ +
D	0·31	0·21	0·46	0·07	+
E	0·31	0·61	0·68	0·22	+
(F)	0·09	0·32	(0·46)	(0·07)	(+)

cannot, after all the precautions taken, be ascribed to experimental errors.

These findings and the following observations pointed to the possibility of existence of a volatile metabolite in 0–12 hr. urine. In some experiments, untreated urine and freeze-dried residues prepared from the same batch did not give identical radioactive recoveries. This difference was not observed in experiments where freeze-dried urine gave a high recovery of radioactivity (Table 1); in Expts. 14 and 15 some samples of urine as collected were also subjected to wet combustion, and the radioactive recoveries were within the range of recoveries obtained with the same freeze-dried urine; but in Expt. 13, where a low radioactive recovery was obtained with freeze-dried urine, untreated urine gave very inconsistent recoveries, ranging from 45 to 70%. In Expt. 5 the variations of radioactive recoveries were also very high (48–74%). A small amount of the same urine sample was freeze-dried and subjected to wet combustion, giving a recovery of only 42% of the total radioactivity. In Expt. 6, however, the recovery of radioactivity in untreated urine was high, with no great variations; a freeze-dried sample also gave the high recovery of 74%.

As the 5-hydroxytryptamine used had ^{14}C at the end of the side chain, it is possible that a volatile compound is formed from the side chain of either 5-hydroxytryptamine or its metabolites. However, even if we accept the idea of the existence of a volatile radioactive compound, this cannot account for such great biological variations in the results obtained.

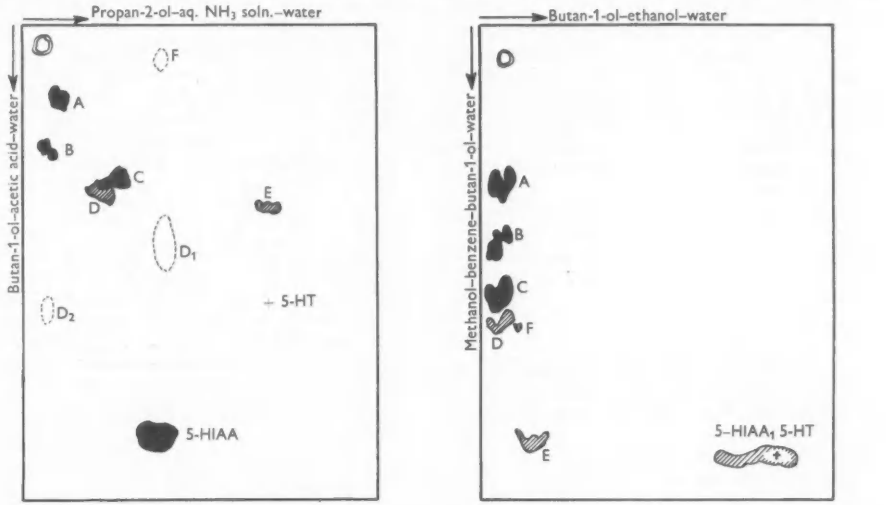

Fig. 3. Radioautographs of two-dimensional chromatograms of 12 hr. urine of rats injected with 5-hydroxy-[1′-^{14}C]tryptamine (5-HT). Before chromatography urine was treated with deactivated charcoal (Dalgliesh, 1955). 5-Hydroxyindolylacetic acid (5-HIAA) and A–F are metabolites; D_1 and D_2 are degradation products. Intensity of spots: black, high; cross-hatched, weak; broken line, very weak; +, position of 5-hydroxytryptamine in untreated urine.

Table 3. *Chromatographic behaviour of 5-hydroxyindolylaceturic acid and its hydrolysis products*

For conditions of hydrolysis see text; for solvent systems see Fig. 2.

	R_F in solvent system				Colour with	
	1	2	3	4	Ehrlich's reagent	Ninhydrin
5-Hydroxyindolylaceturic acid	0·63*	0·35	0·49	0·19	Deep blue	—
	0·09	0·14	0·29	0·12	—	Blue–violet
Products of hydrolysis	0·77	0·33	0·61	0·57	Blue	—
Glycine†	0·06	0·12	0·25	0·04	—	Blue–violet
5-Hydroxyindolylacetic acid†	0·80	0·33	0·61	0·53	Blue	—

* Value given by McIsaac & Page (1958) is 0·84.

† Run on the same sheet with hydrolysis products.

All radioactive metabolites of 5-hydroxytryptamine detected in urine were rather unstable compounds. The positive test with Ehrlich's reagent indicates the presence of the indole nucleus. The carbazole and naphtharesorcinol tests suggest that compounds A and D are glucuronides. Compounds A and B showed a very low oxytocic activity. In the urine of rats pre-treated with iproniazid, the amount of compound A increased considerably at the expense of 5-hydroxyindolylacetic acid (our unpublished data). From these findings compound A seems to be identical with 5-hydroxytryptamine *O*-glucuronide, identified by Weissbach *et al.* (1958) in the urine of mice pre-treated with iproniazid.

5-Hydroxyindolylaceturic acid as a metabolite of 5-hydroxytryptamine was discussed by Erspamer (1955), and by Udenfriend, Titus, Weissbach & Peterson (1956), and was claimed to be found in carcinoid urine by McIsaac & Page (1958). We could not detect this conjugate in rats' urine. The R_F given by McIsaac & Page (1958) for this compound is not in accordance with the R_F of synthetically prepared 5-hydroxyindolylaceturic acid (Table 3). The R_F of a second metabolite of 5-hydroxytryptamine, 1′-*N*-acetyl-5-hydroxytryptamine, in butanol–acetic acid–water (our solvent system 1), given by the same authors does not correspond to the R_F of any radioactive spots obtained in these experiments. Radioactive indican would not be expected to be formed from 5-hydroxytryptamine with ^{14}C in the side chain.

SUMMARY

1. 5-Hydroxy[1′-^{14}C]tryptamine creatinine sulphate at a dose of 2·9 mg./100 g. body wt. (1·27 mg. of the free base/100 g. body wt.) was injected subcutaneously into 15 rats.

2. The outstanding feature of metabolism of 5-hydroxytryptamine was a rapid elimination of radioactivity within 12 hr.; the radioactivity excreted varied from 36 to 82% of the dose. In all experiments 12–24 hr. urine had a low radioactivity, corresponding to about 4% of the injected dose.

3. Besides 5-hydroxyindolylacetic acid, the major metabolite of 5-hydroxytryptamine, six radioactive compounds of unknown structure were found in 12 hr. urine. All of them gave a positive test with Ehrlich's reagent and two of them are believed to be glucuronides. A small amount of unchanged 5-hydroxy[1′-^{14}C]tryptamine was recovered in urine.

4. The synthesis and properties of 5-hydroxyindolylaceturic acid are described. This compound was not detected as a metabolite of 5-hydroxytryptamine in rats.

The authors are indebted to Professor D. Hadži and Dr S. Detoni of the University of Ljubljana for the infrared spectra and to Dr L. Filipović and his staff (this Institute) for the microanalysis. We would also like to acknowledge the valuable technical assistance given to us by Mrs D. Orlić.

REFERENCES

Amin, A. H., Crawford, T. B. B. & Gaddum, J. H. (1954). *J. Physiol.* **126**, 596.
Bray, H. G., Humphris, B. G., Thorpe, W. V., White, K. & Wood, P. B. (1952). *Biochem. J.* **52**, 412.
Calvin, M., Heidelberger, C., Reid, J. C., Tolberg, B. H. & Yankwich, P. F. (1949). *Isotopic Carbon.* New York: Wiley and Sons Inc.
Chadwick, B. T. & Wilkinson, J. H. (1958). *Biochem. J.* **68**, 1 P.
Coward, K. H. (1947). *The Biological Standardization of the Vitamins*, 2nd ed., p. 15. London: Baillière, Tindall and Cox.
Curzon, G. (1957). *Arch. Biochem. Biophys.* **66**, 497.
Dalgliesh, C. E. (1955). *J. clin. Path.* **8**, 73.
Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
Erspamer, V. (1955). *J. Physiol.* **127**, 118.
Erspamer, V. (1956). *J. Physiol.* **133**, 1.
Goldschmidt, S. & Wick, M. (1952). *Liebigs Ann.* **575**, 217.
Keglević-Brovet, D., Kveder, S. & Iskrić, S. (1957). *Croat. Chem. Acta*, **29**, 351.
Keglević-Brovet, D., Supek, Z., Kveder, S., Iskrić, S. & Kečkeš, S. (1958). *Proc. 2nd U.N. int. Conf. Peaceful Uses of Atomic Energy, Geneva*, **15**, 481.
McIsaac, W. M. & Page, I. H. (1958). *Science*, **128**, 537.
Moore, A. M. & Boylen, J. B. (1953). *Science*, **118**, 19.
Stoll, A., Troxler, F., Peyer, J. & Hofmann, A. (1955). *Helv. chim. acta*, **38**, 1452.
Udenfriend, S., Titus, E. & Weissbach, H. (1955). *J. biol. Chem.* **216**, 499.
Udenfriend, S., Titus, E., Weissbach, H. & Peterson, R. E. (1956). *J. biol. Chem.* **219**, 335.
Van Slyke, D. D. & Folch, J. (1940). *J. biol. Chem.* **136**, 509.
Weisburger, J. H., Weisburger, E. K. & Morris, H. P. (1952). *J. Amer. chem. Soc.* **74**, 2399.
Weissbach, H., Redfield, B. G. & Udenfriend, S. (1957). *J. biol. Chem.* **229**, 953.
Weissbach, H., Redfield, B. G. & Udenfriend, S. (1958). *Fed. Proc.* **17**, 418.

The Effect of Hypophysectomy of the Rat and of Treatment with Growth Hormone on the Incorporation of Amino Acids into Liver Proteins in a Cell-free System

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Removal of the pituitary gland from an animal results in inhibition of growth, and treatment with growth hormone can, in suitable circumstances, cause a hypophysectomized animal to increase in body weight (see Ketterer, Randle & Young, 1957). However growth is defined (Weiss, 1955), protein synthesis must be intimately concerned in the process, and the implication of the many experiments with hypophysectomized animals is that certain of the secretions of the pituitary gland, especially growth hormone, influence protein synthesis. The liver, among other organs of the rat, decreases in size and protein content as a result of hypophysectomy, and this fall can be prevented to a large extent by treatment with growth hormone (Lee & Schaffer, 1934; Simpson, Evans & Li, 1949). Siekevitz (1952) and Zamecnik & Keller (1954) have shown that rat liver can be used as the source of a cell-free system which will incorporate amino acids into protein *in vitro*. Evidence is accumulating which suggests that many of the reactions studied with this system are concerned in protein biosynthesis *in vivo* and, indeed, that protein synthesis may be occurring to a small extent in this cell-free system. This paper describes the investigation, in the rat, of the effect of hypophysectomy, and of treatment with growth hormone, on the incorporation of amino acids into protein *in vitro* in the cell-free system of Zamecnik & Keller (1954).

METHODS

Animals. Female albino rats were used and in some experiments they were allowed to eat freely of a mixed diet. Because the amount of food eaten influences the rate of protein synthesis, in most experiments the rats were housed in individual cages and each given the same ration of a powdered diet (Diet 41, Bruce & Parkes, 1949) made up into a thick pellet with water. The daily ration, established by trial and error, was the largest quantity of food which would be entirely consumed by every rat of an experimental group. The rats were kept on this ration (about 10 g. of diet 41/100 g. of rat/day) for at least 10 days before treatment with hormones was begun. Hypophysectomy, by the para-pharyngeal method (Ingle & Griffith, 1942), was carried out at least three weeks before the animals were used in an experiment, completeness of removal of the pituitary being checked by observation at autopsy. A sham operation was carried out on the control rats used in some of the experiments.

Growth hormone. Ox growth hormone was used and was prepared by the method of Wilhelmi, Fishman & Russell (1948). It was dissolved in aq. 0·9% NaCl at pH 8·0 and the appropriate dose was always injected subcutaneously in 0·5 ml. of NaCl solution. Control rats were similarly given 0·5 ml. of aq. 0·9% NaCl.

Radioactive compounds. Generally (G) labelled L-[G-^{14}C]-leucine and DL-[1-^{14}C]leucine were used in most of the experiments, but some results were obtained with DL-[1-^{14}C]valine, DL-[β-^{14}C]tryptophan and hydrolysed algal protein labelled with ^{14}C. These radioactive compounds were obtained from The Radiochemical Centre, Amersham, Bucks.

Materials. The sodium salts of adenosine triphosphate (ATP) and of guanosine triphosphate were obtained from the Sigma Chemical Co. The sodium salt of creatine phosphate was prepared by the method of Ennor & Stocken (1948) and creatine phosphokinase was prepared by the method of Kuby, Noda & Lardy (1954).

Preparation of the cell-free system. The method was that of Zamecnik & Keller (1954). Rats were killed by decapitation and were bled. The liver was rapidly removed and cut up with scissors into an ice-cold homogenizing medium consisting of 0·03 M-$KHCO_3$, 0·025 M-KCl, 0·35 M-sucrose and 0·01 M-$MgCl_2$ in 0·02 M-KH_2PO_4–K_2HPO_4 buffer at pH 7·5. The 0·01 M-$MgCl_2$ was the last solution added during the preparation of the medium, which was gassed for 1 hr. with O_2+CO_2 (95:5) before use. The pH was measured with a glass electrode standardized with a standard buffer solution made up, in accordance with the instructions of the manufacturers, from Burroughs Wellcome and Co.'s N.B.S. buffer solution tablets. The pH of the standard solution was 6·9 at 25°. A portion of the liver was blotted, weighed by displacement in ice-cold medium and homogenized in 3 vol. of the medium in a hand-operated homogenizer of the type described by Dounce, Witter, Monty, Pate & Cottone (1955), which was kept cold in an ice bath. Only three strokes (up and down) were used since severe damage to the tissues diminishes its capacity to incorporate amino acids *in vitro* (Zamecnik & Keller, 1954).

The resulting brei was spun at 15 000 *g* for 10 min. at 0° in a refrigerated centrifuge to remove unbroken cells, cell debris, nuclei and mitochondria, and the supernatant fluid, which is referred to as the cell-free system and which contains the microsomes and the soluble fraction of the cell, was used as quickly as possible after preparation.

Separation of microsomes and soluble fraction. The supernatant fluid obtained by centrifuging at 15 000 *g* could be separated into microsomes and the soluble fraction of the

cell by spinning at 105 000 *g* in a Spinco Model L preparative ultracentrifuge for 1 hr. at 0°. The microsome pellet was rinsed in the medium, suspended by gentle homogenization in fresh medium and made up to a known volume. Great care was taken to standardize rigidly the method of preparation of the cell-free system, of the microsomes and of the soluble fraction, so that they could be prepared in exactly the same way from livers of differently treated groups of rats.

Conditions of incubation. Samples of the cell-free system, or of the microsomal and soluble fractions containing the required amount of protein, were mixed with ATP solution containing equimolar $MgCl_2$, and with creatine phosphate solution, so that the final concentration of ATP and creatine phosphate were 6 and 23 mM respectively. The radioactive amino acids, dissolved in the medium, were added to obtain a final concentration of 0·13 mM (about 0·4 μc/ml. of final vol.). The total volume of the mixture was adjusted to 1 ml. with the buffered salt medium. The pH of all solutions was adjusted to 7·5 with 0·1 N-KOH before they were added to the mixture. The reaction was stopped by the addition of cold 0·5 N-$HClO_4$, or, if the microsomes were to be removed from the soluble fraction, the contents of the tubes were poured into cooled Spinco centrifuge tubes containing ice-cold 0·25 M-sucrose together with an excess of unlabelled amino acid, and the tubes centrifuged at 105 000 *g* for 1 hr. The proteins of the soluble fraction and of the resuspended microsomes were then precipitated with cold 0·5 N-$HClO_4$.

Preparation of a freeze-dried system. Recently Sachs (1957) has found that the cell-free system prepared from rat liver may be freeze-dried and stored at −20° for some months without loss of the ability to incorporate amino acids into proteins *in vitro*. Some of the experiments described in this paper were carried out with material prepared and kept in such a fashion.

The whole liver was excised as before, cut up into ice-cold 0·25 M-sucrose, and homogenized in the way described above. The brei was then centrifuged at 15 000 *g* for 10 min. at 0°, and the supernatant fluid shell-frozen in acetone–CO_2, freeze-dried and stored at −15°. For an experiment this material was dissolved, in centrifuge tubes, in a medium consisting of 0·03 M-2-amino-2-hydroxymethylpropane-1:3-diol hydrochloride (tris) buffer at pH 7·5 containing 0·08 M-KCl, 0·05 M-NaCl, 5 mM-$MgCl_2$ and 2·5 mM-glutathione (GSH). The pH was measured with a glass electrode standardized against a standard buffer solution. Microsomes and soluble fraction were separated from the freeze-dried material in the same way as with fresh tissue (see above) and the microsomes suspended by gentle homogenization in the tris medium. Material prepared in this way was shaken at 37° for 30 min. in the presence of mM-ATP, 20 mM-creatine phosphate, 0·6 mM-guanosine triphosphate, 0·03 mg. of creatine phosphokinase and 0·13 mM radioactive amino acid (about 0·4 μc) in a total volume of 1 ml. The reaction was stopped by the addition of either cold 0·5 N-$HClO_4$ or ice-cold sucrose containing excess of unlabelled amino acid, and the microsomes were removed as described above.

Preparation of radioactive proteins. The precipitated protein was washed three times with cold 0·5 N-$HClO_4$ containing excess of unlabelled amino acid to remove radioactive amino acid. Ribonucleic acid (RNA) was removed by extraction with N-NaOH at room temperature for 1 hr., after which the mixture was acidified with 6N-HCl (Scott, Fraccastoro & Taft, 1956). The residual material was boiled with 0·5 N-$HClO_4$ and extracted twice at room temperature with a chloroform–ethanol–ether mixture in the ratios of 1:2:2 (by vol.), in order to remove lipids, and the powder was then dried with ether.

Weighed aluminium planchets, 5 cm.² in area, were screwed into the caps of 4 oz. screw-capped jars. The jars had been cut off at the bottom of the neck so as to leave a short column of glass above the cap. A slurry of the dry protein powder was made with a mixture of ether and light petroleum (1:1, v/v) and this slurry was poured on to the planchet in its bottle top and the solvent allowed to evaporate. In this way 1–15 mg. of protein powder can be evenly plated onto the planchet. The planchets were then reweighed, and the radioactivity of the protein was estimated by counting with a Geiger–Müller end-window counter (1·5–2·5 mg./cm.²). Sufficient counts were recorded to reduce the standard error to 3% or less, and corrections were made for the background counts and for self-absorption. The results are given as the specific activity of the protein (counts/min./mg. of protein).

Statistical treatment of results. In those experiments where enough material could not be obtained from one liver, the livers of several rats which had been treated in the same way were pooled and the cell-free system was prepared from the pooled material. In every experiment replicate samples from the liver of one rat, or pooled livers of a group of rats, were incubated under each of the conditions studied and the mean value of the specific activities of their protein was calculated and considered to constitute one experimental observation. Each experiment was repeated several times with the livers from rats kept under identical conditions, and the mean of the experimental observations, together with the standard error of their means (S.E.M.), calculated. Student's *t* test was applied to ascertain whether the difference between means was statistically significant, and values of Fisher's *P* are given when a difference between mean values is considered to be statistically significant.

Determination of ribonucleic acid. Ribonucleic acid (RNA) was determined in samples of microsomal and soluble fractions by a slight adaptation of the method of Scott *et al.* (1956) in which the cold 0·5 N-$HClO_4$ ppt. of the sample was washed with cold 0·5 N-$HClO_4$ and with cold ether–ethanol (1:1, v/v) and then extracted twice for 1 hr. at room temp. with N-NaOH. The mixture was then acidified with 6N-HCl. The RNA content of the combined alkaline extracts was determined from the extinction at 260 mμ in the Beckman spectrophotometer by using an extinction coefficient of 34·2/mg./ml./cm. (Scott *et al.* 1956).

Determination of protein content. The nitrogen content of samples of the fractions incubated was estimated by the micro-Kjeldahl technique. Corrections were applied for the nitrogen content of any tris buffer present and of the RNA in the samples (by using 14% as the nitrogen content of RNA) and the protein content of the samples was calculated from this corrected nitrogen-content figure by using the factor 6·25. These data were used in some experiments to calculate the specific activity of the microsomal protein by the expression $SPX/100M$, where S is the specific activity of the total protein isolated, P is the weight (mg.) of protein incubated, M is the weight (mg.) of microsomal protein incubated and X is the percentage of the total

radioactivity in the protein of the sample which is found to be present in the microsomal protein fraction. X was determined by removing the microsomes from the soluble fraction in two or three of the samples at the end of each of the incubation periods, and determining the specific activity of the protein of each fraction. This observed value of X was then used to calculate the specific activity of the microsomal protein in the rest of the samples. Because the number of samples was very large any attempt to divide them all into microsomes and soluble fraction at the end of the experiment was precluded, and in any case the error introduced by assuming that the value of X could be applied to all the samples of one type in an experiment was found by trial to be very small. If the results presented have been calculated by use of this formula a note to this effect appears in the legend to the Table or Figure concerned.

Determination of glycogen. The glycogen content of the liver was determined by a modification of the method of Good, Kramer & Somogyi (1933).

RESULTS

Incorporation of amino acid into protein in the cell-free system. Fig. 1 shows the results of a typical experiment in which the cell-free system (i.e. the whole supernatant fluid after centrifuging at 15 000 *g*) is incubated with DL-[1-^{14}C]leucine under the conditions described. The specific activities of the proteins of the cell-free system, prepared from the livers of normal rats, are compared with the specific activities of the proteins of the cell-free system, prepared in exactly the same way from the livers of hypophysectomized rats, after various periods of incubation under identical conditions.

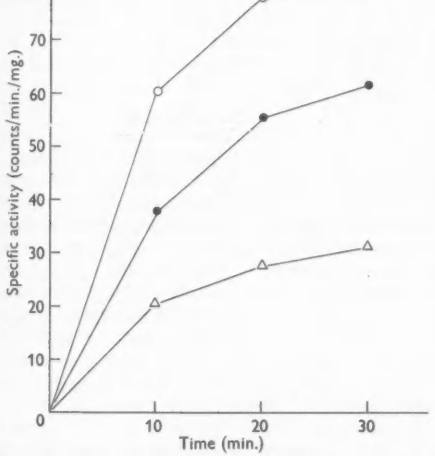

Fig. 1. Specific activities of the proteins of cell-free systems after incubation at 37° for various times with DL-[1-^{14}C]leucine. Protein (8–10 mg.) from the livers of normal (○), hypophysectomized (△) or hypophysectomized rats treated with 0·2 mg. of growth hormone/day for 5 days (●) were incubated in 0·02 M-KH_2PO_4–K_2HPO_4 buffer (pH 7·5), containing 0·03 M-$KHCO_3$, 0·025 M-KCl, 0·35 M-sucrose and 0·01 M-$MgCl_2$, together with 6 mM-ATP, 23 mM-creatine phosphate and 0·13 mM-DL-[1-^{14}C]leucine (0·4 μc) in a total vol. of 1 ml.

It can be seen that amino acid is rapidly incorporated into protein and that a plateau level of specific activity is reached in about 20 min. in both instances, but that much less amino acid is incorporated into the proteins of the preparation from the livers of hypophysectomized rats than into the proteins of the liver preparation from normal rats. Many experiments of this type have been carried out; whenever the conditions of the experiment led to the optimum incorporation of amino acid by the proteins of the cell-free preparation from normal rats, the specific activity of the protein found in the companion preparations from hypophysectomized rats was about 40% or less of the value found for preparations from normal animals. In earlier experiments, when a search was being made for the optimum conditions of preparation and incubation of the cell-free system, the conditions differed somewhat from those described above and the specific activity of the proteins of the cell-free system from normal rat liver was less than the values indicated in Fig. 1. But in all such instances the amino acid incorporated into the protein from hypophysectomized animals was less than that with preparations from normal rats, ranging from 20 to 70% of that found with the protein from normal rats (Korner, 1958).

Treatment of the hypophysectomized rats with ox growth hormone made possible the preparation of a cell-free system which incorporated more amino acid into protein *in vitro* than did the system obtained from the untreated hypophysectomized rats. Fig. 1 shows the incorporation of DL-[1-^{14}C]-leucine into protein of liver preparations from hypophysectomized rats given 0·2 mg. of growth hormone/day for 5 days. In no instance was treatment with ox growth hormone alone successful in restoring the extent of incorporation of amino acid by the cell-free preparation from hypophysectomized rats to that of preparations from normal rats.

When microsomes and soluble fraction are prepared from the cell-free system by centrifuging at 105 000 *g*, the amino acid-incorporating system has been separated into two parts, neither of which alone incorporates amino acids to any great extent when shaken at 37° in the presence of ATP, creatine phosphate, creatine phosphokinase, guanosine triphosphate and radioactive amino acids.

Table 1. *Incorporation in a cell-free system of* DL-[1-^{14}C]*leucine into microsomal protein prepared from livers of normal and hypophysectomized rats*

3 mg. of microsomal protein and 4 mg. of soluble-fraction protein were incubated for 30 min. at 37° in 0·03 M-tris buffer (pH 7·5) containing 0·08 M-KCl, 0·05 M-NaCl, 5 mM-$MgCl_2$, 2·5 mM-GSH, mM-ATP, 0·02 M-creatine phosphate, 0·6 mM-guanosine triphosphate, 0·03 mg. creatine phosphokinase and 0·13 mM-DL-[1-^{14}C]leucine (0·4 μC) in a total vol. of 1 ml. The specific activity of the microsomal protein was calculated from the formula given in the Methods section.

Source of protein fractions incubated		Specific activity of microsomal protein (mean ± S.E.M.) (counts/min./mg.)
Microsomes	Soluble fraction	
Normal rats	—	48 ± 7·3
—	Normal rats	4 ± 1·3*
Normal rats	Normal rats	187 ± 10·1
Hypophysectomized rats	Hypophysectomized rats	71 ± 6·0
Hypophysectomized rats	Normal rats	78 ± 5·2
Normal rats	Hypophysectomized rats	178 ± 9·2

* Counts/min./mg. of soluble-fraction protein.

More amino acid is incorporated into microsomal protein incubated alone than into soluble fraction protein incubated alone under these conditions, presumably because the microsomal pellet traps some of the soluble fraction so that some amino acid can be activated by the amino acid-activating enzymes present in the soluble fraction of the cell (Hoagland, Keller & Zamecnik, 1956), and some amino acid-soluble ribonucleic acid intermediate (Hoagland, Zamecnik & Stephenson, 1957) can be formed (see Discussion). Incorporation of amino acid to the same extent as in the unseparated system does occur when both microsomes and soluble fraction are incubated together, and it can be seen from Table 1 that when microsomes from normal rats were incubated with soluble fraction from normal rats the mean specific activity of the microsomal protein was 187 counts/min./mg. The mean specific activity of the protein of microsomes from hypophysectomized rats, after incubation with soluble fraction from hypophysectomized rats, was 38% of that of the protein from normal rats. The difference between these means is statistically highly significant ($P < 0·001$). When microsomes from hypophysectomized rats were incubated with soluble fraction from normal rats the specific activity was almost the same as when microsomes and soluble fraction, both from hyophysectomized rats, were incubated together, showing that alterations in the soluble fraction from livers of hypophysectomized rats were not responsible for the diminution in incorporation of amino acid by the preparation from hypophysectomized rats. Again, incubation of microsomes from normal rats with soluble fraction from hyophysectomized rats resulted in almost the same specific activity as when microsomes and soluble fraction from normal rats were incubated together. In other words, the soluble fraction from the livers of hypophysectomized rats is just as able as the soluble fraction from the livers of normal rats to prepare amino acids for incorporation into protein, at least to the extent required by the microsomes from normal rats. The diminished incorporation of amino acid into protein found in the complete preparation from the livers of hypophysectomized rats must be caused by some alteration in the microsomes. Any alteration of the ability of the soluble RNA to form a complex with the amino acid and to transfer it to the microsomal particle, which may result from hypophysectomy, cannot be the limiting factor during incorporation of amino acid into protein in this system.

Table 2. *Incorporation of* DL-[1-^{14}C]*leucine into microsomal protein in cell-free systems prepared from livers of two normal rats*

3 mg. of microsomal protein and 4 mg. of soluble-fraction protein were incubated with 0·13 mM-DL-[1-^{14}C]-leucine (0·4 μC) at 37° for 30 min. Conditions of incubation are given in the legend to Fig. 1.

Source of the protein fractions incubated		Specific activity of microsomal protein (counts/min./mg.)
Microsomes	Soluble fraction	
Rat *A*	Rat *A*	162
Rat *B*	Rat *B*	154
Rat *A*	Rat *B*	152
Rat *B*	Rat *A*	149

Similar results to those described in Table 1, which were carried out with freeze-dried material, were obtained in experiments with material which had not been freeze-dried.

Table 2 gives the results of an experiment which was designed as a check of the accuracy of the method. From two samples of liver (*A* and *B*), one from each of two normal rats, the microsomes and soluble fraction were prepared in the way described. It can be seen from the table that the specific

Table 3. *Specific activity of microsomal protein from rat liver after 30 min. incubation in the cell-free system with* L-[G-^{14}C]*leucine after various preincubation procedures*

The conditions of incubation are as in Fig. 1. Procedure 1: no preincubation; procedure 2: preincubation of whole homogenate for 15 min. at 37°; procedure 3: preincubation of microsomes and soluble fraction together for 15 min. at 37°; procedure 4: preincubation of microsomes and soluble fraction separately for 15 min. at 37°. The counts/min./mg. of microsomal protein are calculated from the formula given under Methods.

	Counts/min./mg. of microsomal protein			
Treatment of microsomes ...	Procedure 1	Procedure 2	Procedure 3	Procedure 4
Treatment of soluble fraction				
Procedure 1	165	158	136	72
Procedure 2	10	12	—	—
Procedure 3	87	—	58	—
Procedure 4	132	—	—	67

activity of the microsomal protein from preparation *A* and from preparation *B*, after incubation under the usual conditions, was approximately the same whether the microsomes from *A* were incubated with the soluble fraction from *A* or from *B* or whether the microsomes from *B* were incubated with soluble fraction from *A* or *B*.

Possibility of degradation of the microsomes in vitro. It seemed possible that the microsomes of hypophysectomized rats are initially just as able as those from normal rats to incorporate amino acids into protein *in vitro*, but that this ability falls off rapidly during the experiment and results in a difference in the amount of amino acid incorporated by the two types of microsome over a period of time. The possible rise in the rate of breakdown could be caused by a greater activity of proteolytic or of ribonuclease enzymes in preparations from hypophysectomized rats than in those from the normal rat, or by a greater sensitivity to the activity of such degradative enzymes of the microsomes from hypophysectomized rats than of those from normal rats. Roth (1954) has indeed suggested that the ribonuclease (RNAase) enzymes of rat liver may be concerned in protein synthesis. An experiment was carried out to test the possibility that the difference found between the microsomes of normal and hypophysectomized rat liver might result from a greater breakdown of the microsomes from hypophysectomized rats' liver under the conditions of these experiments.

Homogenates from the livers of normal and of hypophysectomized rats were incubated at 37° for various times before centrifuging at 15 000 *g* to remove nuclei and mitochondria, and Fig. 2 shows the subsequent incorporation of radioactive leucine into the proteins of the cell-free systems prepared from these preincubated homogenates. It is clear that in both instances rapid inactivation has taken place during preincubation. When, however, microsomes from a preincubated homogenate were incubated with soluble fraction prepared from a liver homogenate which had not been preincubated,

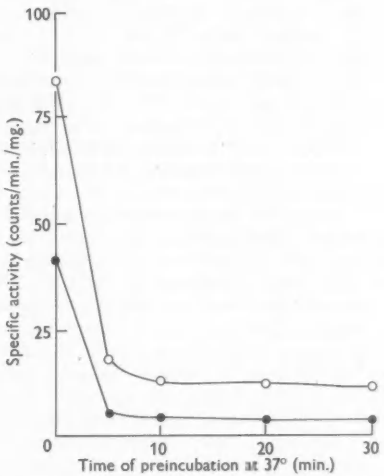

Fig. 2. Specific activities of the proteins of the cell-free systems after incubation with DL-[1-^{14}C]leucine for 30 min. at 37°. The systems were prepared from livers of normal (○) and hypophysectomized (●) rats after preincubation of the whole homogenate at 37° for various times. The conditions of incubation are as in Fig. 1.

almost normal rates of incorporation of amino acid into microsomal protein were observed. The results obtained when microsomes which had not been preincubated were incubated with soluble fraction which had been preincubated confirmed that it was indeed mainly the soluble fraction that was being altered by preincubation in these experiments, and not the microsomes. Table 3 presents these results, and also those of an experiment in which the preincubation was carried out at different stages in the preparation of the microsomes and soluble fraction. Four procedures were used. In procedure 1 the microsomes and soluble fraction were prepared without preincubation; in procedure 2 the whole homogenate was incubated at 37° for 15 min.

before the microsomes and soluble fraction were separated from the rest of the liver brei. In procedure 3 the homogenate was centrifuged at 15 000 *g*, to remove debris, nuclei and mitochondria, the supernatant fluid preincubated for 15 min. at 37° and then the microsomes and the soluble fraction were separated from one another by centrifuging at 105 000 *g*. Finally, in procedure 4 the soluble fraction and a suspension of the microsomes in the buffered medium were prepared and then incubated separately at 37° for 15 min.

Table 3 shows that a large diminution in incorporation of amino acid into protein results from procedure 2. The microsomes were not greatly affected by this preincubation, but the soluble fraction lost much of its ability to prepare amino acids for incorporation. The ability of the microsomes to incorporate amino acids was depressed to a greater extent by procedure 3 than by procedure 2, but the soluble fraction was less affected by procedure 3 than by procedure 2. Procedure 4 caused the greatest depression in the incorporating ability of the microsomes and the least inhibitory effect on the soluble fraction.

The presence of the soluble fraction during preincubation thus appears to exert a protective influence on the microsomes, but the presence of other cell fractions enhanced the inhibition suffered by the soluble fraction during preincubation. Sachs (1957) has evidence that the soluble fraction protects the microsomes against freeze-drying as well as against preincubation.

The diminution in incorporating ability suffered by a suspension of the microsomes in buffered medium when preincubated alone at 37° (procedure 4) is depicted in Fig. 3, and it is clear from the results that the effect of preincubation on microsomes from hypophysectomized rats is no greater than the effect on microsomes from normal rats.

Optimum conditions for incorporation. Sachs (1957) has shown that the concentration of ATP, creatine phosphate and GSH in the incubation mixture, and the concentration of protein in the microsomal and soluble fractions, are important factors in determining the amount of amino acid incorporated into protein in his freeze-dried system. Experiments have now been carried out with this system which show that the optimum concentrations of ATP, creatine phosphate and GSH are not greatly different for preparations from normal and from hypophysectomized rats, but that there are different optimum concentrations of the protein fractions.

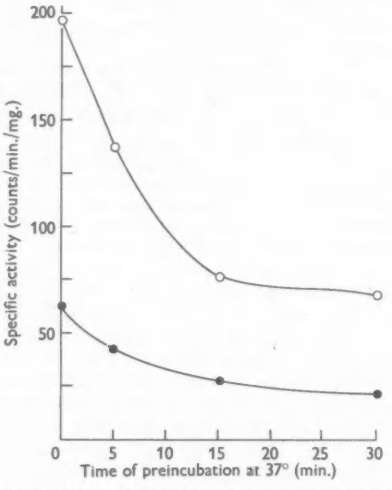

Fig. 3. Specific activities of microsomal protein from livers of normal (○) and hypophysectomized (●) rats after incubation with DL-[1-^{14}C]leucine at 37° for 30 min. under the standard conditions (see legend to Fig. 1). The microsomes had been preincubated at 37° in the buffered medium for the periods shown.

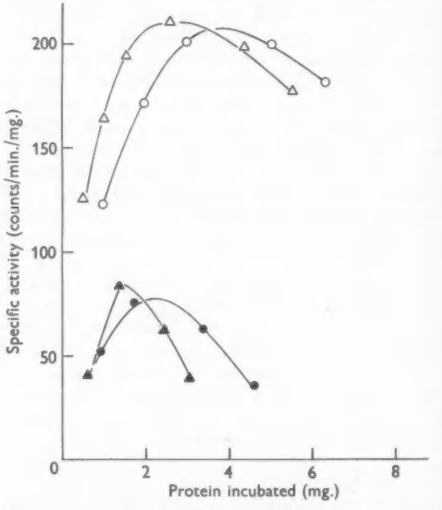

Fig. 4. Specific activities of microsomal protein from the livers of normal (○, △) and hypophysectomized (●, ▲) rats after incubation for 30 min. at 37° with L-[G-^{14}C]-leucine under the standard conditions. In one experiment (△, ▲) various amounts of microsomal protein were incubated with a constant amount of soluble-fraction protein and in the other (○, ●) the amount of soluble-fraction protein incubated varied while the microsomal protein content was kept constant. The conditions of incubation are as in Table 1.

Fig. 4 shows the effect of incubating different amounts of microsomal protein with a constant amount of soluble-fraction protein, and different amounts of soluble-fraction protein with a constant amount of microsomal protein. An optimum concentration for both microsomal protein and soluble-fraction protein is found with systems prepared from livers of normal and hypophysectomized rats (cf. Sachs, 1957), but the optima occur at lower protein concentrations with the preparations from hypophysectomized rats. Nevertheless, the differences in incorporation between normal and hypophysectomized rats observed cannot be explained only in terms of differences in the optimum concentrations of proteins, since, as is clear from Fig. 4, the incorporation of amino acids into microsomal protein from hypophysectomized rats was less than that into microsomal protein from normal rats even when the concentration of the former was optimum.

The results of these experiments support the view that the difference in the rate of incorporation of amino acids into protein *in vitro* observed between preparations from normal and hypophysectomized rats is not the result of changes in the microsomes which occur after removal of the liver from the animal, nor is it caused by any difference in the conditions needed by the system from hypophysectomized rats to achieve optimum incorporation of amino acids.

Effect of food intake. Hypophysectomized rats eat less than intact ones, and the amount of food eaten daily can influence the rate of amino acid incorporation into protein and the rate of protein biosynthesis. For example, Rutman, Rutman & Tarver (1955) have shown that changes in the diet influence the rate of incorporation of radioactive methionine into liver slices. It is possible that hypophysectomy is not affecting the microsomes directly but that the changes are secondary to changes in food intake.

The experiments described in Table 1 have therefore been repeated with groups of rats all the members of which ate the same amount of the same diet; the results obtained were similar to those obtained with rats which were allowed to eat freely. Differences in food intake between normal and hypophysectomized rats cannot therefore explain the differences in incorporation of amino acid into protein.

Table 4 shows the results of an experiment in which normal and hypophysectomized rats were kept on the same ration for a week and then starved for 18 hr. before preparation of the cell-free system. There is little difference between the starved animals and the others in the amounts of amino acid incorporated into protein. Krahl (1956) believes that the incorporation of amino acid into rat-liver slices from normal and hypophysectomized rats varies directly with the glycogen content of the slices. The glycogen content of the livers of the animals used in the experiments of Table 4 was determined and, as expected, the starving animals, both normal and hypophysectomized, had almost none. Yet in both groups only small differences in incorporation of amino acid were associated with starvation. The glycogen content of the liver appears not to affect the rate of incorporation of amino acids into protein in the cell-free system.

Treatment of rats with growth hormone. Table 5 shows the results of experiments in which female rats, both normal and hypophysectomized, were treated with growth hormone, and the incorporation of amino acid into their microsomal protein was measured in the cell-free system. It is clear that the effect of hypophysectomy on the microsomes can be suppressed, at least in part, by treatment with growth hormone. The earliest significant effect ($P < 0.01$) of growth hormone so far detected was when 0·5 mg. was given 20 hr. before killing the animal. Intravenous injection of growth hormone 1 hr. before death, and subcutaneous injections 3, 6 and 12 hr. before death, produced no significant change in incorporation of amino acids into proteins in the cell-free system. The effect on normal rats of treatment with growth hormone is also shown in Table 5. It is clear from experiments of the type described above that growth hormone can cause increased incorporating ability in normal microsomes. The relevant results are illustrated in Fig. 5. Treatment of normal and hypophysectomized rats with growth hormone appeared to cause a small increase in the ability of the soluble fraction of the liver to prepare amino acids for incorporation into protein in the microsome, but these changes are not statistically significant. In no experiment did treatment of

Table 4. *Specific activity of microsomal protein from normal and hypophysectomized rats which had been fed, or starved for* 18 *hr. after incubation under identical conditions with* L-[G-^{14}C]*leucine for* 30 *min.*

The conditions of incubation are as in Table 1. The specific activities are calculated from the formula given under Methods.

	Specific activity of microsomal protein (mean ± S.E.M.) (counts/min./mg.)
Normal rats	
Fed	133 ± 8·6
Fasted 18 hr.	121 ± 6·1
Hypophysectomized rats	
Fed	59 ± 5·9
Fasted 18 hr.	52 ± 5·1

Table 5. *Effect of various doses of ox growth hormone in female rats on the specific activity of microsomal protein after subsequent cell-free incorporation experiments with* L-[G-^{14}C]*leucine*

The conditions of incubation are as in Table 1. The specific activities are calculated from the formula given under Methods.

	Dose of growth hormone (mg./day)	Time of treatment (days)	Specific activity of microsomal protein (mean ± S.E.M.) (counts/min./mg.)
Hypophysectomized rats	Nil	—	82 ± 5·5
	0·5*	1 hr.	77 ± 6·1
	0·5	1	106 ± 6·0
	0·02	3	116 ± 7·1
	0·2	3	152 ± 10·5
	0·2	7	160 ± 9·9
Normal rats	Nil	—	189 ± 11·2
	0·02	3	196 ± 10·1
	0·2	3	211 ± 11·1
	0·2	7	239 ± 8·9

* Intravenous injection.

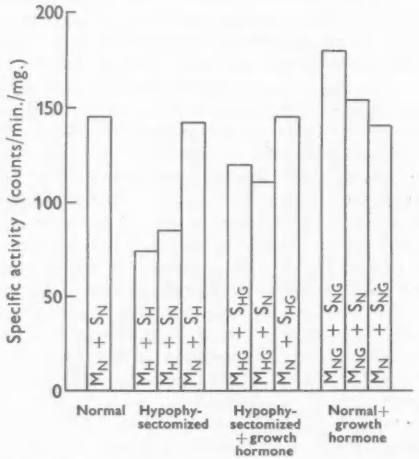

Fig. 5. Histogram showing the specific activity of microsomal protein after incubation for 30 min. at 37° with DL-[1-^{14}C]leucine, of microsomes from normal and hypophysectomized rats with their own and one another's soluble fraction. M_N, microsomes from normal rats; M_H, microsomes from hypophysectomized rats; S_N, soluble fraction from normal rats; S_H, soluble fraction from hypophysectomized rats; the additional subscript $_G$ signifies that the rat was treated with 0·4 mg. of growth hormone each day for 4 days. Conditions of incubation are as in Table 1. Microsomal specific activity was calculated from the formula given under Methods.

hypophysectomized rats with growth hormone alone succeed in completely reversing the depressing effect which hypophysectomy has on the incorporation of amino acids into microsomal protein *in vitro*.

DISCUSSION

Some steps in the sequence of reactions which results in the incorporation of amino acids into microsomal protein are known. The amino acids are activated by specific amino acid-activating enzymes, which exist in the soluble fraction of the cell and which are precipitated at pH 5·0 (Hoagland *et al.* 1956; Nismann, Bergmann & Berg, 1957; Lipmann, 1958). Activation requires ATP, and, since pyrophosphate is eliminated in the process, it is suggested that an amino-acyl adenosine monophosphate complex is formed on the surface of the activating enzyme. Hoagland *et al.* (1957) have good evidence that the next step is the formation of a complex between the activated amino acid and the rather low molecular-weight ($10–20 \times 10^3$) soluble RNA of the cytoplasm. Guanosine triphosphate is needed in the next step, in which the amino acid enters the microsome (Hoagland, Stephenson, Scott, Hecht & Zamecnik, 1958) attached to all or part of the soluble RNA (Crick, 1958). Littlefield, Keller, Gross & Zamecnik (1955) have provided evidence that the assembly of amino acids into polypeptide chains occurs in the microsomal particles (Palade, 1956) of the microsomal fraction. It is postulated (Littlefield *et al.* 1955; Littlefield & Keller, 1957) that the RNA of these particles functions as a template on which the peptide is formed, and schemes can be constructed to account for the way in which the amino acids fall on specific points on the template (Crick, Griffith & Orgel, 1957) so that the finished peptide which peels off the template has a specific amino acid sequence.

Hypophysectomy and amino acid-incorporating ability. It is clear from the experiment described in this paper that hypophysectomy impairs the amino

acid-incorporating ability of the cell-free system and that the main site of the defect is in the microsomes. Any change in the activity of the amino acid-activating enzymes as a result of hypophysectomy is not responsible for the depression in amino acid incorporation found, for the enzymes of the soluble fraction from hypophysectomized rats can work as well as those from normal rat liver if presented with normal microsomes. Similarly a diminution in the ability of the soluble RNA to form a soluble RNA–amino acid intermediate, of the type suggested by Hoagland *et al.* (1957), or a decrease in the ability to transfer the amino acids from the soluble RNA to the microsomal particles, cannot be responsible for the lower rate of incorporation of amino acid found in preparations from the hypophysectomized rats. It is possible that changes have occurred in the activity of these enzymes and carriers, of such a nature that these reactions are not limiting and are not detectable by these experiments. The conditions used in these experiments are such that excess energy is available throughout in a suitable form, so that any effect of hypophysectomy on the formation *in vivo* of ATP for these processes would be masked. Whether or not hypophysectomy brings about changes in cell permeability to metabolites of importance in protein synthesis likewise is not revealed in these experiments. In other words, although the incorporating ability of the microsomes is affected by hypophysectomy in such a way as to reduce the rate at which protein is synthesized, the possibility has not been excluded that other mechanisms, not detected by the system used here, also contribute to the fall in the overall rate of protein synthesis observed directly in the livers of rats deprived of their pituitary gland (Simpson *et al.* 1949).

The results reported here do not support the possibility that the fall in the ability of microsomes to incorporate amino acids into protein which follows hypophysectomy in the rat is caused by a greater degradative change occurring in such microsomes after removal of the liver from the rat. They have also shown that the fall cannot be explained by the alteration in the optimum conditions of the system prepared from the hypophysectomized rat. It is concluded that an effect on the microsomes has occurred *in vivo* as a result of hypophysectomy and that this has reduced the ability of the microsomes to incorporate amino acids into protein.

Effect of amount of food eaten on protein biosynthesis. Hypophysectomized rats eat less than normal ones but this difference in food intake cannot, by itself, account for the diminution in incorporating ability of the microsomes from livers of hypophysectomized rats, for similar differences in incorporating ability between microsomes from livers of normal and hypophysectomized rats were found whether the rats had been fed on the same ration or had been allowed to eat freely. Moreover, starvation for 18 hr. did not materially alter the ability of the microsomes from livers of normal or hypophysectomized rats to incorporate amino acid into protein. Clark, Naismith & Munro (1957) have shown that starvation for 18 hr. of rats, previously fed on a diet containing protein, causes a fall in the RNA content of the liver. The experiment described in Table 4 shows that the incorporating ability of the microsomes is not altered by this treatment. It can be concluded, either that the RNA lost by 18 hr. starvation is not concerned in protein biosynthesis, or that microsomal protein is lost during starvation for 18 hr. at such a rate that the ratio of microsomal protein to reactive microsomal RNA is kept constant. If this occurs, when similar amounts of microsomal protein from fed and starving rats are used in incubation experiments, they will contain similar amounts of reactive microsomal RNA and, therefore, similar amounts of amino acid will be incorporated into protein. The number of microsomes per liver may, however, diminish as a result of starvation.

Effect of liver-glycogen content on protein biosynthesis. Krahl (1956) found that the amount of amino acid incorporated into the protein of rat-liver slices from hypophysectomized rats was proportional to the glycogen content of the slices. Incorporation of amino acid in the cell-free system in the experiments described in this paper is not related directly to the liver-glycogen content, for similar incorporation was found in the microsomes from fed and from starved rats. These results are not necessarily at variance with one another; for, if the glycogen content of the liver is an index of the amount of glucose available for RNA template formation, or for energy production, then it could be argued that in the experiments with cell-free preparations the amount of amino acid incorporated will depend on the preformed template, but that in slices more template could be formed, or more energy made available, during the experimental period.

Effect of growth hormone on protein biosynthesis. It is encouraging that the results of the experiments *in vitro* reported here agree with the expectations one derives from effects which hypophysectomy and treatment with growth hormone are known to have on protein synthesis *in vivo*. Hypophysectomy of the rat is followed by a loss of body weight and of the protein of the carcass (Lee & Ayres, 1936) and treatment with crude (Lee & Schaffer, 1934) or purified (Simpson *et al.* 1949) growth hormone can reverse or prevent this process. Growth hormone can cause retention of nitrogen in many normal animals (see Ketterer *et al.* 1957), and can stimulate

the livers of hypophysectomized rats to synthesize more serum albumin (Ulrich, Tarver & Li, 1954). Russell's (1951, 1955) evidence indicates that growth hormone acts by stimulating protein synthesis rather than by inhibiting protein breakdown, and a similar conclusion was reached by Friedberg & Greenberg (1947) as a result of experiments on the incorporation of radioactive amino acids into protein in the muscles of mice.

In the experiments described here, it is shown that growth hormone is one of the factors determining the ability of the microsomes of rat liver to incorporate amino acids. Complete quantitative reversal of the effects of hypophysectomy was not obtained with growth hormone alone, probably because other hormones of the pituitary gland play some part, directly or indirectly, in the reactions which bring about protein biosynthesis. The nature of the changes in the microsomes which occur as a result of hypophysectomy are being studied.

SUMMARY

1. Less radioactive amino acid was incorporated into protein *in vitro* in a cell-free system (Zamecnik & Keller, 1954) prepared from the livers of hypophysectomized rats than was incorporated into protein in a similar system prepared from the livers of normal rats.

2. It was shown that changes in the activity of the amino acid-activating enzymes of the soluble fraction of the cell or in the ability of the soluble ribonucleic acid to form amino acid–soluble ribonucleic acid intermediates were not responsible for the diminished incorporation of amino acid into protein found in this system as a result of hypophysectomy. The microsomes from the livers of hypophysectomized rats were, however, less able than those from normal rats' liver to incorporate the activated amino acids into protein.

3. The diminished ability of the microsomes to incorporate amino acids into microsomal protein shown by liver preparation from hypophysectomized rats could not be attributed to (*a*) greater breakdown of the microsomes during incubation by more extensive ribonuclease or proteolytic-enzyme activity, (*b*) differences in the optimum conditions needed by the preparations from differently treated rats, or (*c*) the smaller food intake of hypophysectomized rats compared with that of normal rats.

4. Starvation of normal or of hypophysectomized rats for 18 hr. did not alter the ability of the liver microsomes to incorporate amino acids into protein. Incorporation of amino acid into protein in this system is not related to the glycogen content of the liver.

5. Treatment of the hypophysectomized rats with ox growth hormone could restore, in part, the ability of their liver microsomes to incorporate amino acids into protein. Other pituitary hormones may also be concerned in these processes. Treatment of normal rats with growth hormone increased the ability of the liver microsomes to incorporate amino acid into protein.

6. It is concluded that hypophysectomy of the rat results in changes in the liver microsomes such that they are less able to incorporate amino acids into protein than liver microsomes from normal rats and that treatment with growth hormone can partly reverse such changes. In this way the depressed rate of protein biosynthesis observed *in vivo* in the livers of hypophysectomized rats may be at least partly accounted for.

It is a pleasure to thank Professor F. G. Young, F.R.S., for his interest in this work and for his encouragement. I wish to thank the Council of the Royal Society for a Grant-in-aid, Miss M. B. Thomas, who performed the hypophysectomies, and Mr D. C. Gardiner for highly skilled assistance.

REFERENCES

Bruce, H. M. & Parkes, A. S. (1949). *J. Hyg., Camb.*, **47**, 202.

Clark, C. M., Naismith, D. J. & Munro, H. N. (1957). *Biochim. biophys. Acta*, **23**, 587.

Crick, F. H. C. (1958). *Symp. Soc. exp. Biol.* **12**, 138.

Crick, F. H. C., Griffith, J. S. & Orgel, L. E. (1957). *Proc. nat. Acad. Sci., Wash.*, **43**, 416.

Dounce, A. L., Witter, R. F., Monty, K. F., Pate, S. & Cottone, M. A. (1955). *J. biophys. biochem. Cytol.* **1**, 139.

Ennor, A. H. & Stocken, L. A. (1948). *Biochem. J.* **43**, 190.

Friedberg, F. & Greenberg, D. M. (1947). *Arch. Biochem.* **17**, 193.

Good, C. A., Kramer, H. & Somogyi, M. (1933). *J. biol. Chem.* **100**, 485.

Hoagland, M. B., Keller, E. B. & Zamecnik, P. C. (1956). *J. biol. Chem.* **218**, 345.

Hoagland, M. B., Stephenson, M. L., Scott, J. F., Hecht, L. I. & Zamecnik, P. C. (1958). *J. biol. Chem.* **231**, 241.

Hoagland, M. B., Zamecnik, P. C. & Stephenson, M. L. (1957). *Biochem. biophys. Acta*, **24**, 215.

Ingle, D. J. & Griffith, J. Q. (1942). In *The Rat in Laboratory Investigation*, p. 380. Ed. by Griffith, J. Q. & Farris, E. J. Philadelphia: J. P. Lippincott Co.

Ketterer, B., Randle, P. J. & Young, F. G. (1957). *Ergebn. Physiol.* **49**, 127.

Korner, A. (1958). *Nature, Lond.*, **181**, 422.

Krahl, M. E. (1956). *Recent Progr. Hormone Res.* **12**, 199.

Kuby, S. A., Noda, L. & Lardy, H. A. (1954). *J. biol. Chem.* **209**, 191.

Lee, M. O. & Ayres, G. B. (1936). *Endocrinology*, **20**, 489.

Lee, M. O. & Schaffer, N. K. (1934). *J. Nutr.* **7**, 337.

Lipmann, F. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 67.

Littlefield, J. W. & Keller, E. B. (1957). *J. biol. Chem.* **224**, 13.

Littlefield, J. W., Keller, E. B., Gross, J. & Zamecnik, P. C. (1955). *J. biol. Chem.* **217**, 111.

Nismann, B., Bergmann, F. H. & Berg, P. (1957). *Biochem. biophys. Acta*, **26**, 639.

Palade, G. E. (1956). *J. biophys. biochem. Cytol.* **2**, 85.
Roth, J. S. (1954). *Nature, Lond.*, **174**, 129.
Russell, J. A. (1951). *Endocrinology*, **49**, 99.
Russell, J. A. (1955). In *Hypophyseal Growth Hormone, Nature and Actions*, p. 213. Ed. by Smith, R. W., jun., Gaebear, O. H. & Long, C. N. H. New York: McGraw-Hill.
Rutman, J., Rutman, R. J. & Tarver, H. (1955). *J. biol. Chem.* **212**, 95.
Sachs, H. (1957). *J. biol. Chem.* **228**, 23.
Scott, J. F., Fraccastoro, A. P. & Taft, E. B. (1956). *J. Histochem. Cytochem.* **4**, 1.
Siekevitz, P. (1952). *J. biol. Chem.* **195**, 549.
Simpson, M. E., Evans, H. M. & Li, C. H. (1949). *Growth*, **13**, 151.
Ulrich, F., Tarver, H. & Li, C. H. (1954). *J. biol. Chem.* **209**, 117.
Weiss, P. (1955). In *Hypophyseal Growth Hormone, Nature and Actions*, p. 3. Ed by Smith, R. W., jun., Gaebler, O. H. & Long, C. N. H. New York: McGraw-Hill.
Wilhelmi, A. E., Fishman, J. B. & Russell, J. A. (1948). *J. biol. Chem.* **176**, 735.
Zamecnik, P. C. & Keller, E. B. (1954). *J. biol. Chem.* **209**, 337.

Incorporation of Sodium [1-^{14}C]Hexanoate and Sodium Hydrogen [^{14}C]Carbonate into Milk Constituents by the Perfused Cow's Udder

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The occurrence of a C_6 volatile acid was observed in the ox rumen by McClymont (1951) and in the sheep rumen by Gray, Pilgrim, Rodda & Weller (1952). The metabolism of [1-^{14}C]hexanoate and [2-^{14}C]hexanoate in the intact dairy cow was studied by Kleiber and his collaborators. Most of the ^{14}C activity, recovered in milk constituents, was present in the lactose, smaller quantities of ^{14}C being found in the fat and casein. The specific activity of plasma glucose, after injection of [1-^{14}C]hexanoate, was much higher than that of acetate. It was concluded from this observation that the transfer of carbon derived from hexanoate to glucose mostly bypasses the plasma acetate pool, even though acetate can contribute carbon to plasma glucose. A similar conclusion was reached from the labelling of plasma acetate and glucose after injection of ^{14}C-labelled butyrate (Kleiber, 1958).

In the experiments described here we have studied the incorporation of sodium [1-^{14}C]hexanoate into different constituents of milk and tissue by the perfused cow's udder. The results indicate that the udder has the capacity to degrade hexanoate to acetyl-coenzyme A. This hexanoate does not behave in a glycogenic manner.

In a previous experiment it was shown that $^{14}CO_2$ was incorporated only to a negligible extent into fatty acids and lactose by the perfused-udder preparation (Cowie *et al.* 1951). No information is available about the incorporation of carbon dioxide into the amino acids of casein by the same preparation. For these reasons a perfusion experiment was performed in the presence of sodium hydrogen [^{14}C]carbonate and the incorporation into amino acids was studied.

EXPERIMENTAL

Perfusion of half-udders. Each experiment was carried out on one lactating half-udder perfused during 150 min. by the method of Peeters & Massart (1952). Half-udders from different cows were utilized. Experimental details of the perfusion technique and of $^{14}CO_2$ collection were as described by Verbeke, Lauryssens, Peeters & James (1959). Immediately before slaughter the cows were milked out as completely as possible with the aid of intravenously injected oxytocin. The udders were removed as soon as the cows were shot. The glands were completely bisected along the median septum and one half was connected to the perfusion apparatus. Heparinized, fresh cow's blood (9 l.) was used for the perfusion of one half.

Sodium [1-^{14}C]hexanoate (0·2 m-mole = 1 mc) was dissolved in 50 ml. of water and added to the blood 30 min. after the beginning of perfusion. The experimental animal had yielded approx. 18 l. of milk daily. After the administration of labelled substrate, inactive acetate (sodium acetate trihydrate, 5·25 g. in 250 ml. of water) was introduced regularly into the blood reservoir by means of a constant-drip device. At the end of the experiment oxytocin (10 i.u.) was injected into the udder artery to cause ejection of milk: 290 ml. of milk was collected. Skin, teats and adipose tissue were removed carefully by dissection. The udder tissue was weighed (wt. 3·88 kg.), cut into pieces and cooled at $-15°$ for 1 hr.

Sodium hydrogen [^{14}C]carbonate (5·9 mg. = 1 mc) was added to a solution of inactive sodium acetate trihydrate (10·5 g. in 750 ml. of water). This solution was added to the blood reservoir by means of a constant-drip device started 30 min. after the commencement of perfusion. The experimental animal had previously yielded approx. 12 l. of milk daily. After perfusion 150 ml. of milk was collected.

Isolation of udder tissue and milk-fat fractions. Udder tissue and milk-fat fractions were isolated after perfusion with [1-^{14}C]hexanoate as described previously (Verbeke *et al.* 1959).

Table 1. *Specific activities of constituents of udder tissue and milk after perfusion with* $NaH^{14}CO_3$ *or* [1-^{14}C]*hexanoate*

Specific activities are given as mμc/mg. of C (±S.D.).

Substance	Udder tissue	Milk
[^{14}C]*Bicarbonate experiment*		
Casein	—	0·020±0·005
[1-^{14}C]*Hexanoate experiment*		
Casein	—	1·15±0·03
Whey proteins	—	0·23±0·03
Lactose	—	0·080±0·005
Volatile fatty acids	3·8±0·1	0·090±0·005
Non-volatile fatty acids	0·95±0·05	0·050±0·005
Phospholipids	0·57±0·05	0·00
Cholesterol	0·070±0·005	0·00
Glycerol	0·00	0·00

Chromatographic separation of fatty acids. The even-numbered straight-chain saturated and unsaturated fatty acids were fractionated chromatographically by the reversed-phase technique of Howard & Martin (1950) and by the vapour–liquid chromatography method of James & Martin (1952), as described previously. The fatty acids studied ranged from acetic acid to stearic acid.

Isolation of milk proteins. Isolation of milk proteins was carried out in both experiments as previously described. The incorporation of ^{14}C into amino acids of casein was measured after chromatographic separation (Hirs, Moore & Stein, 1954) as described previously.

Isolation of several Krebs-cycle acids from milk and udder tissue. Citric acid was isolated from milk in both experiments. Citric acid, fumaric acid and succinic acid were isolated from tissue in the hexanoate experiment only. The methods used for the isolation of citric acid and succinic acid have been described (Verbeke *et al.* 1959).

Fumaric acid was isolated as follows. 40 fractions, eluted from the Dowex-1 column after the citric acid peak, were combined and evaporated. The residue was dissolved in a minimal volume of 35% (v/v) butanol–chloroform and applied to the top of a silica gel column (Isherwood, 1946). Fumaric acid was eluted with 35% (v/v) butanol–chloroform, the emerging fractions being titrated with 0·01 N-NaOH solution. Further purification of the fumaric acid peak fraction was carried out by paper chromatography (Kalbe, 1954). The acid solution was applied on the paper as a streak and the acid was localized after developing with methyl red indicator solution at pH 8·0. After elution with water, fumaric acid was estimated by the spot-area method.

Radioactivity measurements. The radioactivity measurements were made with a windowless flow counter as described previously (Verbeke *et al.* 1959). The magnitudes of the count rates obtained and the thicknesses of materials used are of the same order as described by Verbeke *et al.* (1959). In the tables the activities are recorded as μmc together with the S.D. from the mean from at least triplicate samples.

RESULTS

$NaH^{14}CO_3$ *experiment.* The casein isolated from milk in the bicarbonate experiment was labelled to only a slight degree as compared with that obtained in the hexanoate experiment (Table 1). As can be seen from Table 2, only glutamic acid and aspartic acid showed a measurable activity. In this experiment aspartic acid is slightly more active than glutamic acid.

Citric acid isolated from milk (Table 4) showed a very high specific activity as compared with aspartic acid and glutamic acid isolated from casein.

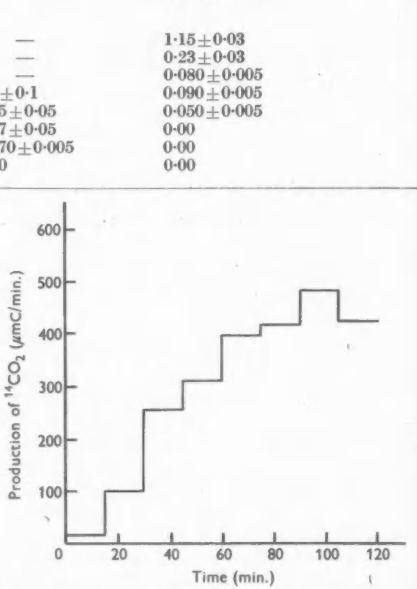

Fig. 1. Production of $^{14}CO_2$ during perfusion in the presence of [1-^{14}C]hexanoate.

Table 2. *Specific activities of amino acids isolated from casein after perfusion with* $NaH^{14}CO_3$ *or* [1-^{14}C]*hexanoate*

Specific activities are given as mμc/mg. of C (±S.D.).

Amino acid	Bicarbonate expt.	Hexanoate expt.
Glutamic acid	0·060±0·005	6·01±0·06
Aspartic acid	0·085±0·005	1·94±0·02
Serine	0·00	0·30±0·01
Alanine	0·00	0·050±0·005
Glycine	0·00	0·085±0·005
Proline	—	0·14±0·01
Valine	—	0·007±0·002

[1-^{14}C]*Hexanoate experiment.* Of the administered ^{14}C 3·6% was recovered as $^{14}CO_2$ liberated by the artificial-lung system. The method used enabled us to collect approx. 80% of the total $^{14}CO_2$. As can be seen from Fig. 1, $^{14}CO_2$ production increased progressively during the experiment to reach a maximum 90 min. after the start of perfusion.

Protein. The highest specific activities in milk were found in citric acid and casein (Tables 1 and 4).

The labelling of amino acids after addition of [1-^{14}C]hexanoate (Table 2) is similar to that obtained in previous perfusion experiments (Verbeke, Aqvist & Peeters, 1957; Lauryssens, Verbeke & Peeters, 1957; Verbeke *et al.* 1959) carried out in the presence of acetic acid, butyric acid and *iso*-valeric acid, showing that appreciable labelling of the non-essential amino acids has occurred. The essential amino acids isolated from casein showed no detectable level of radioactivity. In this experiment the highest specific activity observed was that of glutamic acid, this activity being three times that of aspartic acid.

Fatty acids. The specific activities of the fatty acids isolated from udder-tissue glycerides and milk glycerides are given in Table 3. The specific activity of the acids increases stepwise from butyric acid to octanoic acid and falls progressively from octanoic acid to stearic acid. Both udder and milk fatty acids give the same picture, udder fatty acids from butyric acid to palmitic acid being 25 to 75 times as active as the corresponding milk fatty acids.

The picture of the specific activities of the individual fatty acids is similar to the one obtained after perfusion in the presence of [1-^{14}C]butyric acid and [1-^{14}C]*iso*valeric acid.

About 14% of the ^{14}C dose administered was recovered in the glyceride fatty acid fraction.

Krebs-cycle acids. The specific activities of citric acid, succinic acid and fumaric acid were very high (Table 4). The specific activity of the citric acid fraction isolated from milk is higher than that of citric acid from tissue. The activity of succinic acid and fumaric acid fractions is definitely higher than that of citric acid. Similar results have been obtained recently in several perfusion experiments carried out in the presence of other ^{14}C-labelled precursors ([1-^{14}C]glucose and [6-^{14}C]glucose: unpublished work). It can be calculated that 1·5–2% of the ^{14}C dose added was recovered in citric acid.

Table 3. *Specific activities of even-numbered glyceride fatty acids from udder tissue and milk after perfusion with* [1-^{14}C]*hexanoate*

Specific activities are given as mμc/mg. of C (±S.D.).

Acid	Udder	Milk
Butyric	2·75±0·10	0·058±0·002
Hexanoic	5·10±0·12	0·057±0·003
Octanoic	9·52±0·10	0·132±0·003
Decanoic	8·75±0·10	0·119±0·003
Lauric	7·36±0·10	0·107±0·003
Myristic	1·75±0·06	0·094±0·004
Palmitic	1·45±0·03	0·050±0·002
Stearic	0·020±0·001	0·020±0·005
Palmitoleic [hexadec-7-enoic acid]	0·040±0·002	—
Oleic	0·070±0·003	0·010±0·003

Table 4. *Specific activities of some Krebs-cycle acids from milk and tissue after perfusion with* $NaH^{14}CO_3$ *or* [1-^{14}C]*hexanoate*

	Specific activity (mμc/mg. of C)
[^{14}C]*Bicarbonate experiment*	
Citric acid (milk)	1·12±0·01
[1-^{14}C]*Hexanoate experiment*	
Citric acid (milk)	25·7±0·03
Citric acid (tissue)	15·1±0·04
Succinic acid (tissue)	33·7±1·3
Fumaric acid (tissue)	71·2±1·5

DISCUSSION

The incorporation of sodium [^{14}C]bicarbonate into the amino acids of casein was very small. Only glutamic acid and aspartic acid showed a measurable specific activity. It can be calculated from the production of $^{14}CO_2$ in the presence of [1-^{14}C]-hexanoate that the carbonate pool was involved to only a very small extent in incorporation of ^{14}C into amino acids from [1-^{14}C]hexanoate. The same conclusion is applicable to previous perfusion experiments, in which the incorporation of other precursors, acetate, propionate (Verbeke *et al.* 1957), *iso*valerate (Verbeke *et al.* 1959) and butyrate (Lauryssens *et al.* 1957) into the amino acids of casein was studied. Milk citric acid, obtained from the bicarbonate experiment, showed considerable ^{14}C activity. However, the activity of this constituent in the hexanoate experiment was higher by a factor of 20. It appears that only a negligible fraction of the activity of citric acid in the hexanoate experiment can be attributed to $^{14}CO_2$ fixation. It was shown previously that $^{14}CO_2$ fixation into the fatty acid fractions and into lactose is negligible in the udder-perfusion experiment (Cowie *et al.* 1951). The same conclusion is applicable to casein and citric acid. $^{14}CO_2$ fixation into lactose and casein seems to be a much more important phenomenon in the intact dairy cow. A definite specific activity was noted by Black, Kleiber & Smith (1952) in many essential and non-essential amino acids of casein after intravenous injection of sodium [^{14}C]bicarbonate into lactating cows. It is obvious from these observations that ^{14}C activities in milk constituents are often easier to interpret in udder-perfusion experiments than in the investigations carried out on the intact cow.

Kleiber (1958) noted in the intact cow a considerable incorporation of [^{14}C]hexanoate and [^{14}C]butyrate into lactose of milk. Neither [1-^{14}C]-hexanoate nor [1-^{14}C]butyrate (Lauryssens *et al.* 1957) was acting in a glycogenic manner in the udder-perfusion experiment; in both the specific activity of lactose was very low.

It is evident that [1-^{14}C]hexanoate was broken down by the perfused udder into C_2 components with high acetylating capacity. These components are utilized as precursors of fatty acids and are metabolized by the Krebs cycle, yielding highly labelled citric acid, succinic acid and fumaric acid. The substantial specific activities detected in glutamic acid and aspartic acid from milk casein can easily be explained by transaminase activities. Small amounts of ^{14}C are presumably transferred by way of oxaloacetate to pyruvate, yielding lactose, alanine and serine with low activity. The same picture of ^{14}C incorporation was observed previously in the presence of acetate, butyrate and *iso*valerate.

If hexanoate and butyrate are acting as precursors of carbohydrates in the intact dairy cow but not in the perfused udder it may be supposed that another organ is involved in this conversion. Evidence that a reaction of this kind may occur in the liver of the cow was obtained recently by experiments on the perfused goat liver (Shaw, Lakshmanan, Chung, Leffel & Doetsch, 1958).

There is no evidence of direct esterification of [1-^{14}C]hexanoate, as seen from our results on the distribution of specific activities amongst the fatty acids. The analogy between the metabolism of butyrate and hexanoate in the perfused udder is very striking, both precursors being broken down to C_2 components.

The specific activities of the fatty acids from the udder tissue were 25 to 75 times as great as those found in milk, whereas in milk the casein fraction showed the highest activity. Similar results were obtained frequently in the presence of several precursors: [1-^{14}C]butyrate and [1-^{14}C]*iso*valerate. These results would suggest that hexanoate is acting primarily as a precursor of milk casein. However, this observation may be misleading as there are no data available on the specific activity of the casein in udder tissue. A more reasonable interpretation of these changes in specific activity is that they are a reflexion of the secretory processes in the udder cells. The maximum specific activity of the fatty acids in the intact cow is reached at a later time and the ^{14}C activity is maintained longer than in casein and lactose (Wood 1958).

SUMMARY

1. Perfusions of two halves of lactating cow udders were performed for 2 hr. with blood containing [^{14}C]bicarbonate and [1-^{14}C]hexanoate. Both halves received inactive acetate added continuously throughout the experiments, and 150 and 290 ml. of milk respectively were collected after perfusion.

2. A slight incorporation of $^{14}CO_2$ occurred into milk casein, largely in aspartic acid and glutamic acid. Citric acid isolated from the milk of the same half-udder showed a higher activity.

3. In the hexanoate experiment the specific activity of carbon dioxide was low at the beginning but increased gradually; approximately 4% of the ^{14}C was recovered as $^{14}CO_2$.

4. The specific activities of the milk constituents in the hexanoate experiment decreased in the following order: citric acid, casein, lower fatty acids, higher fatty acids and lactose. Cholesterol, glycerol and phospholipids did not show any detectable activity in milk. Fatty acid fractions isolated from udder tissue were approximately 40 times as active as the corresponding milk fatty acids.

5. The specific activity of the lower fatty acids from udder and milk increased with increasing chain length to reach a maximum at C_8 and then fell progressively with further increase of chain length. In the hexanoate experiment 14% of the added ^{14}C was recovered in fat at the end of the experiment. There was no evidence of direct esterification of [1-^{14}C]hexanoate.

6. Of the activity of milk casein obtained from the [1-^{14}C]hexanoate experiment 97% was due to glutamic acid and aspartic acid, the activity of the former being three times that of the latter.

7. These results are consistent with the assumption that [1-^{14}C]hexanoate is broken down to C_2 components with high acetylating capacity. These components are utilized for the synthesis of fatty acids and are metabolized by way of the Krebs cycle, which is demonstrated by the isolation of citric acid, succinic acid and fumaric acid with high specific activity. Hexanoate did not behave in a glycogenic manner in the perfused udder.

8. The carbonate pool is involved only to a negligible extent in the incorporation of ^{14}C from hexanoate into milk constituents by the perfused udder preparation.

9. The specific activities of the different fractions may be a reflexion of the secretory processes of the udder cells. The fat formed in the cells is secreted in the alveoli at a later time than in the other milk constituents.

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REFERENCES

Black, A. L., Kleiber, M. & Smith, A. H. (1952). *J. biol. Chem.* **197**, 365.
Cowie, A. T., Duncombe, W. G., Folley, S. J., French, T. H., Glascock, R. F., Massart, L., Peeters, G. J. & Popják, G. (1951). *Biochem. J.* **49**, 610.
Gray, F. V., Pilgrim, A. F., Rodda, H. J. & Weller, R. A. (1952). *J. exp. Biol.* **29**, 57.
Hirs, C. H. W., Moore, S. & Stein, W. H. (1954). *J. Amer. chem. Soc.* **76**, 6063.
Howard, G. A. & Martin, A. J. P. (1950). *Biochem. J.* **46**, 532.
Isherwood, F. A. (1946). *Biochem. J.* **40**, 688.
James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.
Kalbe, H. (1954). *Hoppe-Seyl. Z.* **297**, 19.
Kleiber, M. (1958). *2nd U.N. int. Conf. Peaceful Uses of Atomic Energy, Geneva*, paper 812.
Lauryssens, M., Verbeke, R. & Peeters, G. (1957). *Int. Conf. Radioisotop. Scient. Res., Paris*, paper 117.
McClymont, G. L. (1951). *Aust. J. agric. Res.* **2**, 92.
Peeters, G. & Massart, L. (1952). *Arch. int. Pharmacodyn.* **91**, 388.
Shaw, J. C., Lakshmanan, S., Chung, A. C., Leffel, E. C. & Doetsch, R. N. (1958). *2nd U.N. int. Conf. Peaceful Uses of Atomic Energy, Geneva*, paper 813.
Verbeke, R., Aqvist, S. & Peeters, G. (1957). *Arch. int. Physiol. Biochim.* **65**, 433.
Verbeke, R., Lauryssens, M., Peeters, G. & James, A. T. (1959). *Biochem. J.* **73**, 24.
Wood, H. G. (1958). *2nd U.N. int. Conf. Peaceful Uses of Atomic Energy, Geneva*, paper 852.

Parapepsins: Two Proteolytic Enzymes Associated with Porcine Pepsin

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Several groups of workers have reported the presence of other proteolytic enzymes associated with pepsin, either in gastric juice or in extracts of the gastric mucosa. Freudenberg (1940), Pope & Stevens (1951), Buchs (1954) and Taylor (1959) have shown that activity may be found at pH values above 3·0 and this activity has generally been attributed to a cathepsin. These reports are discussed by Taylor (1959). Although Pope & Stevens (1951) were able to obtain a fraction showing a sharp pH optimum at pH 1·7 they were unable to purify a fraction with optimum in the range pH 3·0–4·0. Merten, Schramm, Grassmann & Hannig (1952), by precipitation and electrophoresis, and Richmond, Tang, Wolf, Trucco & Caputto (1958), by passage through a cation-exchange resin, obtained partial separations of the fractions with optimum activity below pH 2·0 and above pH 3·0.

Northrop (1932) obtained a fraction from pepsin which had a gelatin-liquefying activity much greater than that of pepsin.

We have fractionated crude pepsin by ion-exchange chromatography on diethylaminoethyl-cellulose and have isolated two minor components which differ from pepsin in specificity but are similar to it in other properties. One of these enzymes has also been found in a batch of crystalline pepsin.

This paper reports the isolation and characterization of these enzymes; a preliminary account of this work has already appeared (Ryle, 1958).

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EXPERIMENTAL

Abbreviations used in this paper are as follows: acetyl-DL-phenylalanyl-L-di-iodotyrosine, APD; haemoglobin, Hb; dinitrophenyl, DNP; amino acid residues, those used by Brand & Edsall (1947).

Where the activities of the enzymes are expressed as m[P.U.] (defined below) without indication of the substrate, it is to be understood that APD was the substrate for parapepsin I and Hb for parapepsin II; for pepsin the units are equal for the two substrates.

Materials

The crude pepsin used was pepsin B.P. (sold by Hopkin and Williams Ltd., Chadwell Heath, Essex, and manufactured solely from pig gastric mucosae). Several different batches have been used, but no significant difference has been observed between them.

Diethylaminoethyl-cellulose was prepared according to Peterson & Sober (1956) and had about 1 m-equiv. basic groups/g.

Haemoglobin was bovine haemoglobin enzyme-substrate powder (Armour and Co. Ltd., Hampden Park, Eastbourne, Sussex), and was dialysed against water before use.

Acetyl-DL-phenylalanyl-L-di-iodotyrosine was kindly given by Mrs R. V. Pitt-Rivers of this Institute. This stereoisomeric mixture has not been crystallized, but was precipitated five times by acidification of an alkaline solution. It gave no reaction with ninhydrin.

Cysteyltyrosine and tryosylcysteine were also given by Mrs Pitt-Rivers.

Bovine plasma albumin was crystallized material obtained from Armour and Co. Ltd.

The milk powder was Gayelord–Hauser skim-milk powder, obtained locally from Boots Ltd.

Pepsin inhibitor was material obtained from porcine pepsinogen and very kindly sent by Professor R. M. Herriott of Johns Hopkins University.

Gelatin was a sample (no. 141) obtained through the generosity of Mr A. G. Ward, of the British Gelatin and Glue Research Association, and had a viscosity at 6·67%, pH 7·0 and 40° of 6·67 centistokes, and isoelectric point at pH 5·0.

Methods

Instruments. Extinction values were determined with either a Hilger Uvispek or a Unicam spectrophotometer.

pH determinations were made with an E.I.L. (Richmond, Surrey) direct-reading pH meter or a Cambridge pH meter, standardized with Cambridge buffer tablets.

Buffer solutions. Except where otherwise stated, buffers were made from sodium salts. Their concentrations are stated with respect to the anionic component.

Activity determination by haemoglobin. Haemoglobin assays in duplicate were based on the method of Northrop, Kunitz & Herriott (1948), modified by scaling down the volume of each assay to 1·2 ml., to conserve material, and by reading the extinction of the filtrate obtained after adding 5 ml. of 4% trichloroacetic acid (w/v) without dilution, to reduce the labour involved in assaying a large number of chromatographic fractions. The increments in extinction found were converted into milliproteolytic units (m[P.U.]^{Hb}) by reference to a standard curve obtained with a preparation of crystalline pepsin which had been standardized by the original method of Anson (1938). Assays were restricted to the region of near-proportionality of amount of enzyme to extinction.

*Determination of activity by acetyl-*DL*-phenylalanyl-*L*-di-iodotyrosine.* Ninhydrin reagent was freshly prepared each day by mixing equal volumes of 4% (w/v) ninhydrin in 2-methoxyethanol (methylCellosolve) (distilled from $SnCl_2$) and a buffer mixture containing both the acetate buffer (2 M) of Moore & Stein (1954) and the citrate buffer (0·05 M) of the same authors (Moore & Stein, 1948) and 160 mg. of $SnCl_2,2H_2O$/100 ml. The acetate buffer was used because of its high buffering power, but it was found that unless some citrate was added colour yields only about 60% of the theoretical (Troll & Cannan, 1953) were obtained. With the amount of citrate used the colour yield of leucine was 93% of the theoretical and the colour obtained with di-iodotyrosine 90% of the theoretical.

The procedure adopted for the enzymic assay was as follows: to 0·5 ml. of enzyme solution at 35·5° was added 0·25 ml. of HCl of concentration calculated to give finally 0·01 N, followed 10–15 sec. later by 0·25 ml. of 0·002 M-acetyl-DL-phenylalanyl-L-di-iodotyrosine in 0·01 N-NaOH. The mixture was incubated for 20 min., 1·0 ml. of ninhydrin reagent was added and the colour was developed by heating at 100° for 15 min. After dilution with 5 ml. of 60% (v/v) ethanol the extinction at 570 mμ was determined. Assays were performed in duplicate, and duplicate blank determinations were made by adding the ninhydrin reagent to the enzyme before the HCl and substrate and omitting the 20 min. incubation. A standard curve was prepared relating m[P.U.]^{Hb} of pepsin to increase in extinction. This curve was linear to 0·9 m[P.U.]^{Hb} with a slope corresponding to a release of $4{\cdot}1 \times 10^{-3}$ μmoles of di-iodotyrosine/m[P.U.]/min. One m[P.U.]^{APD} has been defined as the activity of one m[P.U.]^{Hb} of pepsin, and the activities of enzyme solutions have been expressed as m[P.U.]^{APD} by reference to this standard curve.

Milk-clotting activity. Determinations of milk-clotting activity were made by the method of Herriott (1938), except that following his present practice (personal communication) a slightly more alkaline buffer (equimolar acetic acid and sodium acetate) was used. The pH of the milk solution was 5·6. [P.U.]^{Ren} is as defined by Herriott (1938) but is not strictly comparable because of the slight difference in pH.

Gelatin-liquefying activity. The change in viscosity of gelatin solution at pH 5·0 produced by the enzymes was determined in conditions as close as possible to those used by Northrop (1932). A sample (15 ml.) of 2·5% (w/v) gelatin solution was mixed with 0·3 ml. of 0·1 N-HCl and 0·3 ml. of enzyme in 0·1 M-acetate buffer, pH 5·6, at $35{\cdot}2 \pm 0{\cdot}1°$ and the viscosity was measured over the following 30 min. in an Ostwald viscometer ($t_{H_2O} = 47{\cdot}3$ sec.). The pH, determined after the digestion, was 4·95. Blank determinations with no enzyme were also performed and gave no change in viscosity. Gelatin-liquefying activity is given as [P.U.]$^{Gel\ V}$ (the percentage fall in the additional viscosity/min.) as calculated by Northrop (1932), but the units may not be comparable with his units because of probable differences in the gelatin used.

Chromatography of crude pepsin. Pepsin B.P. (500 g.) was dissolved in 1 l. of 0·5 N-H_2SO_4, the solution was filtered and the pepsin was precipitated by the addition of 1 l. of saturated $MgSO_4$ solution. The precipitate was washed with $\frac{2}{3}$-saturated $MgSO_4$, suspended in water and brought back into solution by the cautious addition of 0·5 N-NaOH (Northrop, 1930). This solution (about 250 ml.) was then dialysed in the cold against 0·1 M-acetate buffer, pH 5·6, in Visking (Visking Corp., Chicago, Ill., U.S.A.) 24/32 tubing. A small precipitate was then centrifuged off and the supernatant, containing 6–8 g. of pepsin (determined by haemoglobin assay), was run into a column of 50 g. of diethylaminoethyl-cellulose (5 cm. × 35 cm.) previously equilibrated with 0·1 M-acetate buffer, pH 4·0. Under these conditions the pepsin was adsorbed as a faintly visible band at the top of the column. The column was then washed with 0·1 M-acetate buffer, pH 4·0, until the extinction at 280 mμ of the effluent was falling sharply. A gradient of rising NaCl concentration, obtained by running 0·1 M-acetate, pH 4·0, containing 0·2 M-NaCl into a 2600 ml. mixing vessel filled with 0·1 M-acetate, pH 4·0, was then used to elute the adsorbed material from the column. Fractions (45 ml.) were collected from the point at which the gradient was applied and the protein concentration of all the fractions was measured by readings of extinction at 280 mμ. The activities against haemoglobin and acetylphenylalanyl-di-iodotyrosine of alternate fractions in the regions of the extinction peaks were determined.

The materials in the active peaks were pooled separately and concentrated by ultrafiltration through 18/32 Visking dialysis tubing supported by a sleeve of silk net under 60 cm. Hg pressure. Towards the end of this concentration some protein sometimes precipitated but was redissolved on dialysis against 0·1 M-acetate, pH 5·6. The pepsin from these chromatograms was dialysed against water and freeze-dried. All these operations were carried out in the cold room.

Re-chromatography of parapepsins. The concentrated solutions obtained as described above were, in the earlier part of the work, re-chromatographed directly, but in the

later stages parapepsin I was adjusted to pH 6·9 in diethyl barbiturate buffer (0·1 M), allowed to stand for 20 hr. at room temp., returned to pH 5·6 by addition of HCl and dialysed against 0·1 M-acetate, pH 5·6. This treatment at pH 6·9 destroys any contaminating parapepsin II.

The re-chromatography is a scaled-down version of the first (for details see the legend to Fig. 2) so that the gradient of NaCl concentration along the column is nearly the same for all the chromatograms. Suitable fractions were pooled, concentrated by ultrafiltration, dialysed against 0·1 M-acetate, pH 5·6, centrifuged to remove dust, dialysed against ion-exchange water and freeze-dried.

Electrophoresis in starch gel. Electrophoresis of the enzymes in starch gel was performed by the method of Smithies (1955) in 0·05 M-citrate buffer, pH 5·6.

The protein samples (0·08 ml. of solution containing 10 mg. of protein/ml.) were applied to 0·6 cm. × 1·3 cm. segments of Whatman no. 3 MM filter paper and inserted, side by side, into a cut near the middle of a starch block 25 cm. × 4 cm. × 0·6 cm. After the electrophoresis the block was split into upper and lower halves. One half was stained with amidoschwarz 10 B (George T. Gurr, London) but only bands corresponding to the parapepsins could be detected. The other half of the block was stained by an adaptation of the method used by Reindel & Hoppe (1954) for detecting peptides on paper strips. The freshly cut gel was placed in a dish over the HCl–$KMnO_4$ mixture for 5 min. It was then allowed to stand for 5 min. in the air before being immersed in the KI–*o*-tolidine solution. Blue bands appeared, of which those due to parapepsins I and II were in the same position as those detected with amidoschwarz. If the gel was transferred directly from the chlorine atmosphere to the KI a strong blue background was obtained, and the formation of this background was prevented by allowing the gel to stand for 5 min. in the air. Such standing even for long periods was of no avail, however, if the gel was prepared with acetate in place of citrate buffer; apparently the citrate reduces the excess of chlorine and hypochlorite without reducing the labile chlorine bound in the peptide bonds.

Electrophoresis on ethanolysed cellulose. Electrophoresis on ethanolysed cellulose columns was performed by the method of Porath (1956) in 0·05 M-citrate buffer, pH 5·6. The enzyme solutions were applied in 0·5 ml. of water, washed into the column with 0·5 ml. of the citrate buffer and subjected to a potential gradient of about 10 v/cm. The eluate from the column was collected in small (approx. 1 ml.) fractions and analysed, after dilution, for extinction and enzymic activity.

Attempted removal of amino acids and small peptides from parapepsins I and II by ion-exchange resins. A solution of about 20 mg. of the enzyme in water was passed at 1° through a column (0·85 cm. × 9 cm.) of Zeo-Karb 225 and De-Acidite FF (The Permutit Co. Ltd.), both 20–40 mesh, mixed 1:2 (v/v). The effluent was frozen and dried from the frozen state. The recovery of activity from the column was 95% for parapepsin I and 80% for parapepsin II.

Attempted removal of amino acids and small peptides from parapepsins by precipitation with trichloroacetic acid. To 25–30 mg. of the enzyme dissolved in 3 ml. of 0·1 M-acetate buffer, pH 5·6, was added 0·3 ml. of 40% (w/v) trichloroacetic acid. The cloudy solution was then heated at 100° for 5 min. to complete precipitation. The precipitate was washed three times with 4% (w/v) trichloroacetic acid, once with ethanol–ether (1:1, v/v), three times with ether and was then air-dried.

Identification and estimation of N-terminal residues. The *N*-terminal residues of the two parapepsins and of some of the peptides obtained from digests of the *B* chain of oxidized insulin were determined by the fluorodinitrobenzene method. Dinitrophenyl (DNP)-proteins obtained were hydrolysed both with and without the addition of the DNP derivative of the amino acids, previously identified in qualitative experiments as occupying the *N*-terminal position, in order to obtain breakdown-correction factors for these DNP-amino acids. The DNP-amino acids were separated and identified by chromatography on paper in *tert.*-amyl alcohol (Blackburn & Lowther, 1951) and, where necessary, in phosphate buffer (Levy, 1954). The methods used throughout this phase of the work are described in detail by Porter (1957).

Digestion of fraction B *of oxidized insulin.* Insulin (1·3 g.) was oxidized for 20 min. at room temp. with 50 ml. of performic acid [47·5 ml. of 98% formic acid + 2·5 ml. of H_2O_2 (100 vol.), allowed to stand 1 hr.] and was then fractionated by precipitation (Sanger, 1949).

A portion (11 mg.) of the fraction *B* obtained was dissolved in 2 ml. of 0·01 N-HCl, the required amount of enzyme was added in a small volume of buffer and the pH was adjusted to 2·0 by the addition of N-HCl. A trace of thiomersal was added to those mixtures allowed to digest for 20 hr.

Identification of peptides obtained from digests of B *chain of oxidized insulin.* The digests were evaporated to dryness on a rotary still and dissolved in a small amount of formic acid, the solutions were transferred to strips of filter paper (Whatman no. 3 or no. 3 MM) and subjected to electrophoresis in pyridine–acetic acid buffer, pH 6·5 (Ryle, Sanger, Smith & Kitai, 1955). The separated bands were detected with ninhydrin on guide strips cut from the edge of the paper and eluted with N-acetic acid. Samples were hydrolysed (5·7 N-HCl, 105°; 18–20 hr.) and chromatographed two-dimensionally in 80% (w/v) phenol–aq. 0·3% NH_3 soln. and butanol–acetic acid–water (1260:200:540, by vol.) on 36 cm. × 28·5 cm. sheets of Whatman no. 1 paper for identification of the amino acids. In some cases the peptide could be identified from consideration of its constituent amino acids, its electrophoretic behaviour and the known structure of the *B* chain of insulin (Sanger & Tuppy, 1951).

Some of the peptide bands were further fractionated by electrophoresis at pH 3·6 in pyridine–acetic acid buffer (Ryle *et al.* 1955); *N*-terminal residues were identified and estimated by the 1-fluoro-2:4-dinitrobenzene method and non-*N*-terminal amino acids were estimated as their DNP derivatives, separated by two-dimensional chromatography. In some cases *C*-terminal residues were identified by use of carboxypeptidase treated with di*iso*propyl phosphorofluoridate (Harris, 1955). The peptides from the peptic digests were not studied in detail. Their structures have been deduced from their amino acid content and from the results of Sanger & Tuppy (1951) and Brown, Sanger, Smith & Kitai (1955).

RESULTS

Behaviour of the ion-exchange chromatographic system. Fig. 1 shows the changes in pH and chloride concentration (determined by titration with silver

nitrate) occurring in the effluent from a column of smaller size but of the same proportions as those used for separating the enzymes.

Chromatographic separation of the enzymes. Fig. 2 (*A*) shows a typical chromatogram of crude pepsin. No activity against haemoglobin could be detected in any part of the effluent appearing before the gradient was applied, but it was not possible to assay this part of the chromatogram for activity against acetylphenylalanyldi-iodotyrosine because the blank values were too high. At least some of the material eluted before application of the gradient is therefore of relatively low molecular weight.

After the application of the gradient of sodium chloride concentration three active peaks are found. The first to emerge is active against acetylphenylalanyldi-iodotyrosine but has little activity against haemoglobin, and has been named parapepsin I. The second peak, parapepsin II, has little activity against the synthetic substrate but readily digests haemoglobin. The third peak has both activities and is pepsin itself. Although the two parapepsins are well separated from pepsin they are not usually well separated from one another and they have therefore been further purified by a second chromatography in the same system (Fig. 2*B* and *C*).

The parapepsin I, of which the re-chromatography is shown in Fig. 2 (*B*), had been allowed to stand overnight at room temperature at pH 6·9; this treatment, as is shown below, destroys parapepsin II. It nevertheless retains some proteolytic activity against haemoglobin, but the ratio of this activity to the activity against acetylphenylalanyldi-iodotyrosine is only 2·5% of that for pepsin.

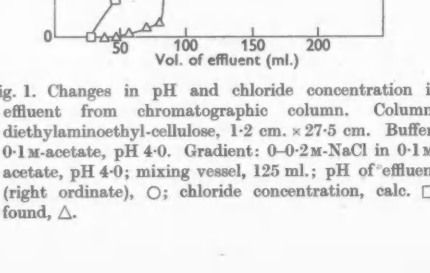

Fig. 1. Changes in pH and chloride concentration in effluent from chromatographic column. Column: diethylaminoethyl-cellulose, 1·2 cm. × 27·5 cm. Buffer: 0·1 M-acetate, pH 4·0. Gradient: 0–0·2 M-NaCl in 0·1 M-acetate, pH 4·0; mixing vessel, 125 ml.; pH of effluent (right ordinate), ○; chloride concentration, calc. □, found, △.

We have not found any such simple treatment by which to free parapepsin II of contaminating parapepsin I, but, by taking suitable cuts from the chromatograms, samples of parapepsin II can be obtained in which the ratio m[P.U.]^{APD}:m[P.U.]^{Hb} is less than 4% of the ratio for pepsin, so that the extent of contamination with parapepsin I or with pepsin cannot exceed 4%, and may be less if parapepsin II has some intrinsic activity against acetylphenylalanyldi-iodotyrosine.

The freeze-dried materials obtained from these and similar chromatograms, or solutions of the same material before freeze-drying, have been used for the studies reported here.

The recovery of the activity against haemoglobin of the solution of crude pepsin at the magnesium sulphate-precipitation step was 70%. In two experiments in which a sample of the filtrate was dialysed and chromatographed no active peak other than that of pepsin was found. It was not possible directly to determine the recovery of parapepsins I and II at the precipitation step, or on

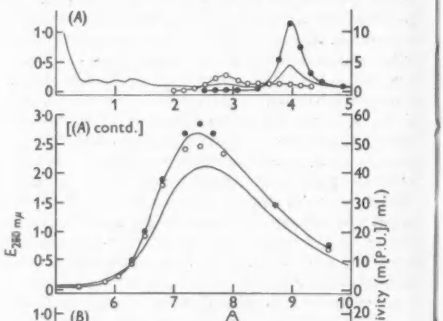

Fig. 2. Gradient-elution chromatography of enzymes on diethylaminoethyl-cellulose. In each the buffer was 0·1 M-acetate, pH 4·0, and the gradient was 0–0·2 M-NaCl in the same buffer. (*A*) Chromatography of crude pepsin. Approx. 6 g. on 5 cm. × 35 cm. column. Mixing vessel 2·6 l. (*B*) Re-chromatography of parapepsin I. 4500 m[P.U.] on 2·4 cm. × 36 cm. column. Mixing vessel 560 ml. (*C*) Re-chromatography of parapepsin II. 5000 m[P.U.] on 2·4 cm. × 40 cm. column. Mixing vessel 560 ml. ○, Activity versus acetylphenylalanyldi-iodotyrosine; ●, activity versus haemoglobin. Experimental points have been omitted from the extinction curve ($E_{280\ m\mu}$) for the sake of clarity.

the first chromatogram, because the high proportion of pepsin present made independent assays of the parapepsins impossible. Assays by acetylphenylalanyldi-iodotyrosine were also not possible at these stages because of the high ninhydrin blank of the crude material.

The recovery of activity of parapepsins I and II on re-chromatography was usually 70–80%, recovery of activity on freeze-drying (relative to extinction) was 95% and the overall yield from 500 g. of crude pepsin has been about 30 mg. of parapepsin I and 90 mg. of parapepsin II. These amounts probably represent about 50% recovery of the parapepsins present in the starting material.

Chromatography of crystalline pepsin. Three batches of commercial crystalline pepsin have been chromatographed on 2·4 cm. × 30 cm. columns of diethylaminoethyl-cellulose. No parapepsin II has been found in any of them, but one batch contained parapepsin I accounting for 4% of the total activity against acetylphenylalanyldi-iodotyrosine.

Specific activities. The specific activities of the parapepsins, measured against their better substrates, are of the same order as that of freeze-dried pepsin obtained from the first chromatogram without re-chromatography (see Table 5). This pepsin itself has 120% of the activity on a weight basis of commercial crystalline pepsin, and taking a nitrogen content for dry, ash-free pepsin of 14·6% (Brand, 1948), and the determined moisture content of freeze-dried pepsin of 14%, the specific activity of 23 $m[P.U.]^{Hb}_{mg.}$ corresponds to 183 $m[P.U.]^{Hb}_{mg.N}$ which compares well with 184 $m[P.U.]^{Hb}_{mg.N}$ found by Anson & Mirsky (1932) for pepsin crystallized five times.

*Product and extent of digestion of acetyl-*DL*-phenylalanyl-*L*-di-iodotyrosine by parapepsin I.* The ninhydrin-positive product recovered from acetylphenylalanyldi-iodotyrosine extensively digested with parapepsin I does not separate from di-iodotyrosine on chromatography in phenol–aq. NH_3 soln., butanol–acetic acid, amyl alcohol–pyridine–water (7:7:6, by vol.) or collidine–water (1000:355, by vol.) in the presence of ammonia vapour. It does not separate either on electrophoresis on paper in N-formic acid, and the same material, eluted from butanol–acetic acid chromatograms and hydrolysed in 5·7N-HCl (105°, 20 hr.), gave no spot corresponding to phenylalanine on paper chromatograms. It is concluded that this material is di-iodotyrosine and that parapepsin I hydrolyses the phenylalanyldi-iodotyrosine peptide bond in the synthetic substrate.

When the progress of digestion was followed by ninhydrin estimations on the whole digest at suitable time intervals it was found that digestion had practically ceased when 46% of the di-iodotyrosine had been liberated. The enzyme apparently hydrolyses only one of the diastereoisomers, presumably the L-L, but no attempt has been made to determine which is in fact hydrolysed.

Electrophoresis of parapepsins I and II. Since these enzymes are bound to diethylaminoethyl-cellulose at pH 4·0 they are apparently acidic proteins, too acid to allow them to be chromatographed on a cation-exchanger such as carboxymethyl-cellulose as a further step in their purification, and in fact it was found that parapepsin I passed unretarded through a column of carboxymethyl-cellulose in 5 mM-acetate buffer, pH 4·5.

To test their purity they were therefore subjected to electrophoresis on a column of ethanolysed cellulose (Porath, 1956) and in starch gel (Smithies, 1955).

On the cellulose column each enzyme appeared as a single symmetrical peak of extinction at 280 mμ, coincident with the peak of enzymic activity. In the starch gel only a single band was found for each enzyme. After electrophoresis for 5 hr. at 2·5v/cm. the bands were about 0·3 cm. wide and had moved from the origin towards the anode as follows: parapepsin I 2·5 cm., parapepsin II 2·9 cm., pepsin 4·7 cm.

Ultracentrifuging of parapepsins I and II. We are indebted to Dr P. A. Charlwood of this Institute, who performed the ultracentrifugal investigations for us in the Spinco model E analytical ultracentrifuge.

The solvent used was acetate buffer, pH 5·6, μ 0·20. Sedimentation patterns showed both parapepsins to be monodisperse ($S_{20,w}$ for parapepsin II 3·32 at 0·3%) and molecular-weight determinations were made by the Archibald (1947) method as used by Charlwood (1957). In a single run parapepsin II gave a set of five values clustered round the mean (40 700), but the values for parapepsin I showed a steady fall with time of centrifuging from 55 000 to 39 000, indicating the presence of some more rapidly sedimented impurity. A 1·1% solution of parapepsin I was therefore centrifuged in the bucket cell at 100 000 *g* (average) for 16 hr. at 2·9–4·7°. The solution in the cell was then carefully removed with a syringe so that three roughly equal fractions, from the top, the middle and the bottom of the cell, were obtained. The middle and lower fractions were re-dialysed against buffer and examined in the analytical cell. The lower fraction gave molecular-weight determinations falling from 63 000 to 43 000 and, on high-velocity sedimentation, a small, rapidly-spreading peak which moved ahead of the main peak in the earlier pictures. This impurity was not more than 2–3% of the total material in the cell.

The middle layer was monodisperse during sedimentation ($S_{20,w}$ 3·26 at 0·42%) and gave a molecular weight (average 38 600) independent of

Table 1. *N-Terminal residues of parapepsins*

In addition to residues shown here traces of the DNP-derivatives of Asp, Glu, Thr, Val and Phe were found. No DNP-glycine was found in samples of the DNP-proteins hydrolysed for 4 hr. No DNP-arginine was found in the aqueous fraction of the 24 hr. hydrolysates.

Treatment	Hydrolysis time (hr.)	Moles found/mole of protein, not corrected for moisture content: Parapepsin I Ala	Parapepsin I Leu/Ileu*	Parapepsin II Ser	Parapepsin II Leu/Ileu*
None	19	0·68	0·19	0·77	0·36
Ion-exchange†	20	0·74	0·12	1·08	0·83
Pptn. with trichloroacetic acid†	24	0·98	0·31	0·78	1·13

* The DNP-derivatives of these amino acids cannot be separated by paper chromatography and we have not determined which isomer is present.
† See Experimental.

Table 2. *Stability of parapepsins*

Each enzyme was diluted in the appropriate solvent to the indicated initial activity. After standing at room temperature (about 20°) for the indicated time the residual activity was determined. Parapepsin I: initial activity 1·3 m[P.U.]/ml. Time, 49 hr. pH 2·0, 0·01 N-HCl; pH 4·0 and 5·6, 0·1 M-acetate; pH 6·9, 0·04-M-diethylbarbiturate. All contained NaCl to give μ 0·10. Parapepsin II: initial activity 0·75 m[P.U.]/ml. Time, 98 hr. pH 2·0, 0·01 N-HCl; pH 4·0, 0·1 M-acetate; pH 5·6, 0·05 M-citrate; pH 6·9, 0·04 M-diethylbarbiturate–0·06 M-NaCl.

pH	Percentage of activity remaining: Parapepsin I	Parapepsin II
2·0	87	80
4·0	95	85
5·6	95	94
6·9	100	0

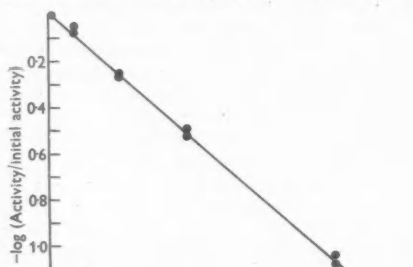

Fig. 3. Inactivation of parapepsin II at pH 6·9, 25°. Initially 7·3 m[P.U.] per ml. 0·04 M-diethylbarbiturate–0·06 M-NaCl.

time of centrifuging, and this fraction had therefore been satisfactorily freed of the heavier components.

N-terminal residues of parapepsins I and II. Preliminary semi-quantitative experiments showed that the *N*-terminal residues of parapepsins I and II were respectively alanine and serine but later experiments showed the presence of *N*-terminal leucine or isoleucine in parapepsin II as well. Table 1 shows the molar proportions of the main DNP-amino acids found, corrected for breakdown on hydrolysis, in three experiments.

Phosphorus content of parapepsins I and II. Phosphorus analyses were made on duplicate 3–4 mg. samples of the two parapepsins by the method of Allen (1940), scaled down to a final volume of 3 ml. Not more than 0·03 g.atom of phosphorus/mole of protein was found.

Stability of parapepsins I and II. Strong solutions of the two parapepsins were diluted in buffers of various pH values and allowed to stand at room temperature. Table 2 shows the percentage of the activity remaining at the times shown. The inactivation of parapepsin II at pH 6·9 is shown in greater detail in Fig. 3; the enzyme has a half-life of 70 min. The difference in stability of parapepsins I and II has been utilized in the purification of parapepsin I (see Methods section: re-chromatography of parapepsins).

A parapepsin II solution with 75% of its activity destroyed by treatment at pH 6·9 does not recover any activity on standing at pH 5·6 or pH 2.

Like pepsin the two parapepsins appear to be rather sensitive to surface denaturation; it has been noticed when pooling chromatographic fractions that a skin of insoluble protein tends to collect on the surface as successive fractions are added and some part of the loss of activity during chromatography is probably due to the same cause.

pH *optima of parapepsins.* The relative initial rates of digestion of acetylphenylalanyldi-iodotyrosine by parapepsin I are plotted against pH in Fig. 4.

Fig. 5 shows the progress of digestion of haemoglobin at three pH values by parapepsin II and by pepsin. The peptic digestion proceeds smoothly to an extinction well beyond 0·6, but with parapepsin

II the rate of reaction falls at E 0·5 and then follows a linear course for some time. The relative slopes of these linear phases of the reaction at these and some other pH values are plotted against pH in Fig. 4 as 'later reaction'. By using a lower concentration of enzyme the initial rate of digestion was measured at different pH values. These relative initial rates are also plotted in Fig. 4.

It can be seen that the pH optima for parapepsin I and for the later part of the digestion of haemoglobin by parapepsin II lie close to pH 1·8, whereas the optimum for the initial digestion of haemoglobin is at pH 3·0. Experiments in which the pH has been adjusted only by the addition of sodium hydroxide to acid-denatured haemoglobin show that in the absence of buffer salts the initial digestion of haemoglobin by parapepsin II is still 1·3 times as fast at pH 3·0 as it is at pH 3·5.

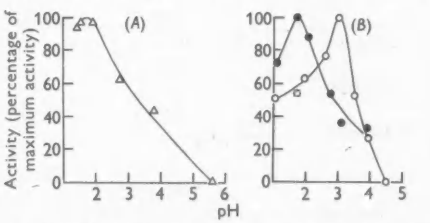

Fig. 4. pH optima of parapepsins at 35·5°. (*A*) Parapepsin I on acetylphenylalanyldi-iodotyrosine, 1·3 m[P.U.]/ml. At pH 3·80 and 5·62 the mixture contained 0·05 M-acetate, at pH 2·75 0·05 M-citrate; lower pH values were obtained with HCl only. All solutions contained NaCl (to μ 0·10) and 0·5 mM-acetyl-DL-phenylalanyl-L-di-iodotyrosine. (*B*) Parapepsin II on haemoglobin. ○, Initial reaction 0·083 m[P.U.]/1·2 ml.; ●, later reaction 0·33 m[P.U.]/1·2 ml. All digestion mixtures were made from 15 ml. of 2% haemoglobin (acid-denatured) + 3·0 ml. of (enzyme + buffer + HCl or NaOH). At pH 3·0 and 3·5 the mixture contained 0·02 M-citrate, at pH 3·9 and 4·5 0·01 M-acetate. At lower pH values no buffer was present.

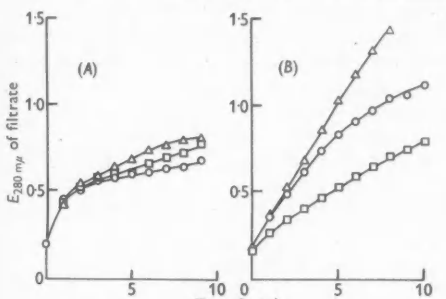

Fig. 5. Digestion of haemoglobin by parapepsin II and pepsin at 35·5°. (*A*) Parapepsin II 0·33 m[P.U.]/1·2 ml. (*B*) Pepsin 0·46 m[P.U.]/1·2 ml. For details of solutions see parapepsin II, Fig. 4(*b*). □, pH 1·1; △, pH 1·7; ○, pH 3·1.

Digestion of bovine plasma albumin. The digestion of bovine plasma albumin produced in 10 min. with varying amounts of pepsin and the two parapepsins up to 0·4 m[P.U.]/1·2 ml. was studied in mixtures made up as in the haemoglobin assay except that haemoglobin was replaced by plasma albumin.

Parapepsin I produced almost no digestion. Parapepsin II showed a linear relationship between increase in extinction at 280 mμ and amount of enzyme (ΔE_{280} 0·19/m[P.U.]). Pepsin was more active; the increase of extinction with 0·2 m[P.U.] was 0·135 but increased with increasing amount of enzyme slightly more rapidly than in a linear relationship.

Digestion of cysteyltyrosine and tyrosylcysteine. Harington & Pitt-Rivers (1944) showed that pepsin would digest cysteyltyrosine and tyrosylcysteine, though only slowly. An attempt was made to determine whether either parapepsin would digest these substrates: digests made with the two enzymes were chromatographed on paper in butanol–acetic acid in the presence of *N*-ethylmaleimide (Hanes, Hird & Isherwood, 1950). However, even after digestion for 24 hr. at 35·5° at pH 2·0 or 4·0 with an enzyme : substrate ratio of 1:10 (w/w), no free tyrosine or cysteine could be detected in digests made with either enzyme.

Effect of thiol and thiol-binding reagents. Pre-incubation of parapepsin II with 20 mM-thioglycollate and mM-ethylenediaminetetra-acetic acid in 0·1 M-acetate, pH 5·6, at 35·5° for 30 min. did not alter the activity of the enzyme (due allowance being made for the increase in the blank occurring on incubation of the haemoglobin substrate alone under the same conditions).

Similarly, pre-incubation of parapepsin I or II with mM-*N*-ethylmaleimide or with 0·1 mM-*p*-chloromercuribenzoate in 0·1 M-acetate, pH 5·6, at 35·5° for up to 2 hr. produced no change in the activity of either enzyme against acetylphenylalanyldi-iodotyrosine and haemoglobin respectively.

Milk-clotting activity and inhibition by pepsin inhibitor. Both parapepsins were found to clot milk, but less readily than does pepsin. The relationship between milk-clotting activity and activity against the substrates used for the regular assay is shown in Table 5. Preincubation of parapepsin II with the pepsin inhibitor (10μg./66μg. of parapepsin II) at 35·5° for 20 min. produced 35% inhibition of the milk-clotting activity of the enzyme. No such inhibitory effect on parapepsin I could be detected with inhibitor:enzyme ratios up to 1:5.

Gelatin-liquefying activity of parapepsins at pH 5. The gelatin-liquefying activities of parapepsins I and II relative to their activities against acetylphenylalanyldi-iodotyrosine and haemoglobin respectively and the same data for chromatographed pepsin are shown in Table 5. Assuming parapepsins I and II to have the same water content (14%) and nitrogen content (14·6%, Brand, 1948) as pepsin these figures can be converted into 3900, 520 and 17 $m[P.U.]^{Gel\,V}_{mg.\,N}$ for parapepsin I, parapepsin II and pepsin respectively.

Sites of attack on the B *chain of oxidized insulin.* Fig. 6 shows the peptide bands, detected by ninhydrin, found on paper-electrophoresis patterns of digests of the *B* chain of oxidized insulin made with pepsin and the two parapepsins under conditions of extensive and of mild digestion. The peptides found (Table 3) in corresponding electrophoretic bands from digests made by the three enzymes are, as far as has been determined, the same. The digests differ in the relative amounts of the different peptide bands found.

Comparison of the data of Fig. 6 and Tables 3 and 4 for the three enzymes allows several conclusions to be drawn. The lack of precise specificity of pepsin is found also with the two parapepsins, which attack the same bonds in the insulin *B* chain as pepsin does, though with a change in the relative rates so that the parapepsins are somewhat more selective than pepsin. From examination of the

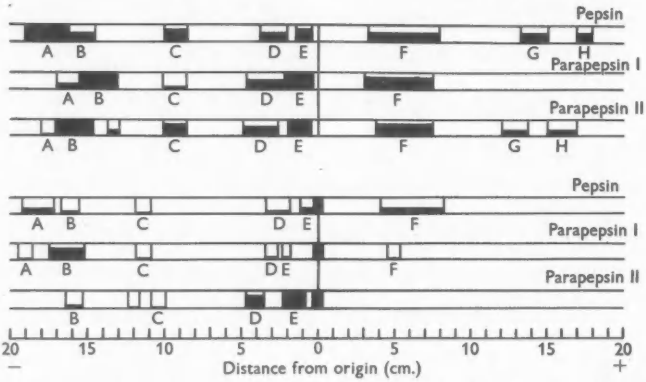
Pepsin
A B C D E F G H
Parapepsin I
A B C D E F
Parapepsin II
A B C D E F G H
Pepsin
A B C D E F
Parapepsin I
A B C D E F
Parapepsin II
B C D E
20 15 10 5 0 5 10 15 20
− Distance from origin (cm.) +

Fig. 6. Peptide bands found on electrophoresis of digests of the *B* chain of oxidized insulin. All digests 0·17 m[P.U.] of enzyme + 5 mg. of *B* chain/ml. Upper group: 20 hr., 36°. Lower group: 3 hr., 20°. Electrophoresis was in pyridine-acetic acid, pH 6·5; 33 v/cm.; 2·5 hr. The strength of the ninhydrin colour of the band is represented by the blackened area on the diagram.

Table 3. *Peptides found in digests of the* B *chain of oxidized insulin*

Peptides from the digests made with parapepsin I and parapepsin II are indicated by the prefixes I and II. Where the presence or absence of terminal residues has not been conclusively demonstrated they are placed in square brackets.

Band	Structure of peptide
A	Tyr.Thr.Pro.Lys.Ala. IIA not certainly identified
B	Phe.Tyr.Thr.Pro.Lys.Ala. IB and IIB have only one Phe per molecule
C	Phe.Val.Asp(NH_2).Glu(NH_2).His.Leu.CySO_3H.Gly.Ser.His.Leu. IC and IIC: Phe.Val.Asp(NH_2).Glu(NH_2).His.Leu.CySO_3H.Gly.Ser.His.[Leu.Val.]
D	Leu.Val.CySO_3H.Gly.Glu.Arg.Gly.Phe.Phe.Tyr.Thr.Pro.Lys.Ala. ID: *N*-terminal Leu. Probably one Leu per molecule. IID: Leu *N*-terminal. Trypsin digestion gives: Leu.Val.CySO_3H.Gly.Glu.Arg, Gly.Phe.Phe.Tyr.Thr.Pro.Lys and Ala
E	Phe.Val.Asp(NH_2).Glu(NH_2).His.Leu.CySO_3H.Gly.Ser.His.Leu.Val.Glu.Ala and Phe.Val.Asp(NH_2).Glu(NH_2).His.Leu.CySO_3H.Gly.Ser.His.Leu.Val.Glu.Ala.Leu. IE: Mostly the longer peptide. Much Leu liberated by carboxypeptidase. IIE: Mostly the shorter peptide. Find 2 Leu per molecule but some Leu liberated by carboxypeptidase
F	Leu.Val.CySO_3H.Gly.Glu.Arg.Gly.Phe, Tyr.Leu.Val.CySO_3H.Gly.Glu.Arg.Gly.Phe and related peptides (cf. Brown, Sanger, Smith & Kitai, 1955)
G	Val.Glu.Ala.Leu. IIG: Leu.Val.Glu.Ala?
H	Val.Glu.Ala

Table 4 *Peptide bonds of the* B *chain of oxidized insulin hydrolysed by pepsin, parapepsin I and parapepsin II*

⇑, Bonds split very rapidly; ↑, other important sites of action; ↑, bonds split slowly.

Bonds split by pepsin (Sanger & Tuppy, 1951):

	Phe	Val	Asp(NH_2)	Glu(NH_2)	His	Leu	$CySO_3H$	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	$CySO_3H$	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Ala
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Bonds split by parapepsin I:											↑			↑	↑	↑								⇑		↑				
Bonds split by parapepsin II:											↑			⇑	↑	⇑								↑		↑				

Table 5. *Summary of properties of pepsin and parapepsins I and II*

For definitions of units of enzymic activity see Experimental Methods.

Substrate for assay	Parapepsin I APD	Parapepsin II Hb	Pepsin Both
$m[P.U.]_{mg.}$	40	35	23
$S_{20,w}$	3·26	3·32	3·16*
Mol.wt.	38 600	40 700	34 500†
N-Terminal amino acid	Ala	Ser + Leu/Ileu‡	Ileu§
Phosphorus (g. atom/mole)	0·03	0·0	1·0‖
Stability at pH 6·9	Stable	Unstable	Very unstable¶
pH optima	1·8	1·8, 3·0	1·5–2·0
Digestion of bovine plasma albumin	−	+	+ + +
$[P.U.]^{Rea}/m[P.U.]$	0·12	0·47	1·2
Inhibition by pepsin inhibitor	−	+	+**
$[P.U.]^{Gel\ V}/m[P.U.]$	12·1	2·5	0·15

* Dieu (1957).
‡ Isomer not identified.
§ Van Vunakis & Herriott (1957); Heirwegh & Edman (1957).
‖ Northrop (1930).
** Herriott (1941).
† Average value. Van Vunakis & Herriott (1957).
¶ Steinhardt (1937).

peptides produced under mild hydrolysis it can be seen that parapepsin I splits the phenylalanyl–phenylalanine (*B*24–*B*25) bond most readily whereas parapepsin II directs its major attack to the alanyl–leucine (*B*14–*B*15) and tyrosyl–leucine (*B*16–*B*17) bonds (numbers in parentheses indicate the position of the peptide bond in the *B* chain).

Examination of the peptides produced by the more prolonged digestion shows some peptides liberated by pepsin which are not produced, or are produced much more slowly, by the parapepsins. Thus both parapepsins form Phe.Tyr.Thr.Pro.Lys.Ala (band B) as the main fragment from the *C*-terminal end of the chain whereas pepsin gives rise mainly to Tyr.Thr.Pro.Lys.Ala (band A). The parapepsins do not readily attack this phenylalanyl–tyrosine bond (*B*25–*B*26). Although parapepsin I produces band E it does not give rise to the peptide in band C in any quantity, nor does it form bands G and H. These differences indicate that this enzyme does not readily hydrolyse the leucyl–valine (*B*11–*B*12) bond, which is hydrolysed by pepsin and probably by parapepsin II.

DISCUSSION

The curves shown in Fig. 1 illustrate a danger inherent in gradient-elution methods; even when changes of salt-concentration are made gradually in the eluting buffer the nature of the ion-exchanger is such that sudden changes of ion concentration and of pH may occur in the effluent. The changes shown in Fig. 1 must arise from the displacement of acetate bound to the DEAE-cellulose by chloride from the eluting buffer, and they could lead to spurious effects in the elution diagram. In the chromatograms presented here the rise in pH would merely lead to a stronger adsorption of the anionic proteins on to the diethylaminoethyl-cellulose, so that the proteins would move more slowly until the wave of raised pH had passed. However, the proteins with which we are concerned do not emerge (Fig. 2) until the volume of effluent exceeds 1 hold-up volume plus 0·68 times the volume of the mixing vessel, whereas the pH change and rapid change of chloride concentration are over before 1 hold-up volume plus 0·58 times the volume of the mixing vessel has passed. The proteins therefore emerge in a normal part of the gradient.

Three kinds of evidence for the purity of parapepsins I and II has been obtained. Electrophoresis, either on a column of ethanolysed cellulose or in starch gel, did not reveal any secondary components; the results with the starch gel are particularly satisfactory as the method of staining, with chlorine, might be expected to detect small peptides, or peptides which did not contain aromatic amino acid residues which might escape detection by amidoschwarz staining or measurements of extinction.

The ultracentrifugal analyses show that parapepsin II is homogeneous whereas parapepsin I has a small amount of material which sediments more rapidly and which is itself heterogeneous. However, this material did not represent more than 2–3% of the total protein even in the lower layer of the ultracentrifuge cell into which it had been concentrated.

The results of the *N*-terminal analyses showed that the enzymes were contaminated with material which could be partly removed by ion-exchange resins or by precipitation of the protein with trichloroacetic acid. Since the enzymes are proteolytic it is not unlikely that they should bind small peptides or amino acids at their active centres, and a high molar ratio of such contaminating peptides would, of course, represent only a very small contamination on a weight basis. That pepsin can bind peptides in this way is indicated by the fact that whereas pure pepsin migrates to the anode in 0·1N-hydrochloric acid, in the presence of digestion products it migrates to the cathode at pH 1·5 (Herriott, Desreux & Northrop, 1940).

These results, taken in conjunction with the absence of phosphorus from the parapepsins and the difference in their relative rates of hydrolysis of haemoglobin and acetylphenylalanyldi-iodotyrosine, indicate that the proteins have been obtained free from pepsin and one another and nearly free of other protein contaminants.

The parapepsins are strikingly similar to pepsin in some properties but nevertheless show important differences in others. These relationships are summarized in the data shown in Table 5. Pepsin is a very acidic protein (Herriott *et al.* 1940) and the two parapepsins behave as anions at pH 5·6 on electrophoresis and at pH 4·0 on chromatography. Their electrophoretic and chromatographic behaviour suggests that they are less acid than pepsin, parapepsin I being the least acid of the three, and this difference accords well with the fact that the parapepsins contain no phosphorus whereas pepsin contains 1 residue of phosphate/mol.

In their molecular weights and sedimentation constants the two parapepsins are similar to pepsin, but whereas pepsin (Van Vunakis & Herriott, 1957; Heirwegh & Edman, 1957) and parapepsin I have only one *N*-terminal residue parapepsin II has two, which suggests that this enzyme must consist of two polypeptide chains or one branched one.

In their stability at pH 6·9, the three enzymes differ markedly. Pepsin is very rapidly inactivated at this pH (Steinhardt, 1937) but parapepsin I is completely stable. Parapepsin II lies somewhere between, being inactivated according to a uni-

molecular reaction (Fig. 3) but more slowly than pepsin. It is perhaps only coincidental that the order of instability of these enzymes at pH 6·9 is the same as that of their mobility at pH 5·6.

Parapepsin I digests acetylphenylalanyldi-iodotyrosine with a pH optimum close to that found for pepsin (Baker, 1951), but parapepsin II on haemoglobin shows two optima: at pH 3·0 for the initial part of the digestion and at pH about 1·8 for the later part. Such a change does not necessarily indicate any heterogeneity of either the enzyme or the protein substrate. It is possible that the peptide bonds most readily hydrolysed are such that the pH optimum is different from that for the digestion of a second group of peptide bonds which are more slowly hydrolysed. In other words, although the protein used as a substrate may be homogeneous it contains a heterogeneous mixture of susceptible peptide bonds which are the true substrates of the enzyme.

Although the parapepsins are clearly differentiated from one another and from pepsin by the differences in their rates of hydrolysis of haemoglobin and of acetylphenylalanyldi-iodotyrosine, consideration of the peptides found in their respective digests of the *B* chain of oxidized insulin indicates that the three enzymes have related specificities, parapepsin I and II each showing part of the activity of pepsin.

In connexion with the observed differences in specificity of the enzymes two points should be noted. First, it must be emphasized that though parapepsin I showed little activity against haemoglobin or bovine plasma albumin this does not mean that no hydrolysis of these proteins occurs. It may be that a few peptide bonds are extensively hydrolysed but that the fragments produced are precipitated by trichloroacetic acid or do not contain tyrosine or tryptophan residues. In either case their formation would not be detected by the method used here, so that it cannot yet be concluded that parapepsin I has little or no activity against protein substrates.

Secondly, it is rather surprising that although parapepsin I hydrolyses the phenylalanyl–diiodotyrosine bond in the synthetic substrate and the phenylalanyl–phenylalanine (*B*24–*B*25) bond in the *B* chain of oxidized insulin it does not cause as much hydrolysis of the phenylalanyl–tyrosine (*B*25–*B*26) bond as pepsin does. This effect may be due not to a fundamentally low rate of hydrolysis of this bond so much as to a relatively greater rate of hydrolysis of the neighbouring phenylalanyl–phenylalanine bond, so that the phenylalanyl–tyrosine bond now lies next to a free amino group which may be inhibitory. It is possible that a phenylalanyl–tyrosine bond found in a different location would be readily hydrolysed.

Both the parapepsins digest casein and gelatin, but at rates widely different from that found with pepsin; however, the most remarkable similarity of specificity is that the milk-clotting activity of parapepsin II is inhibited by pepsin inhibitor, whereas Herriott (1941) found that chicken pepsin was not inhibited by porcine or chicken inhibitor, although the latter would inhibit both bovine and porcine pepsins. The porcine inhibitor was without effect on any of the other proteolytic enzymes tested. Thus parapepsin II is inhibited by the inhibitor hitherto found specific for pepsins of some species only.

The two parapepsins are similar to pepsin in many ways and we must consider whether either of them might be chemically related to pepsin in having a largely common structure. The possibility that either of these enzymes is a fragment of pepsin is eliminated by the results of the molecular-weight determinations, but it is still possible that they may be derived from pepsinogen, which has a molecular weight of 42000 (Herriott, 1938). In this case the peptides removed would have to be smaller than those removed in the activation to pepsin and would have to include the phospate residue which remains with the protein when pepsin is formed. Amino acid analyses of the parapepsins, when available, will help to clarify this question of their possible origin in pepsinogen.

Parapepsin I is similar to the gelatinase of Northrop (1932), in that it liquefies gelatin much more readily than pepsin does, and parapepsin II is similar to the cathepsin of various workers discussed in the introduction, in that its pH optimum for the initial attack on haemoglobin lies above pH 2·0 (although we have not found it to be as high as pH 3·5, the value usually reported for the cathepsin). It is not really possible to relate the gelatinase or the cathepsin to either of the parapepsins, since the first two enzymes have not yet been sufficiently characterized.

SUMMARY

1. Two proteolytic enzymes have been isolated and shown to be minor components of crude pepsin.

2. The enzymes are similar to pepsin in their physicochemical properties, their low pH optimum and their sites of attack on the *B* chain of oxidized insulin. One is inhibited by pepsin inhibitor.

3. The enzymes differ from pepsin in their *N*-terminal residues, their phosphorus content, their stability at pH 6·9 and their activity against various substrates.

4. The possible relationships of these enzymes to pepsin and to other enzymes from gastric sources are discussed.

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REFERENCES

Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Anson, M. L. (1938). *J. gen. Physiol.* **22**, 79.
Anson, M. L. & Mirsky, A. E. (1932). *J. gen. Physiol.* **16**, 59.
Archibald, W. J. (1947). *J. phys. Chem.* **51**, 1204.
Baker, L. E. (1951). *J. biol. Chem.* **193**, 809.
Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **48**, 126.
Brand, E. (1948). Quoted in *Crystalline Enzymes*, 2nd. ed., p. 26. Ed. by Northrop, J. H., Kunitz, M. & Herriott, R. M. New York: Columbia University Press.
Brand, E. & Edsall, J. T. (1947). *Annu. Rev. Biochem.* **16**, 224.
Brown, H., Sanger, F., Smith, L. F. & Kitai, R. (1955). *Biochem. J.* **60**, 556.
Buchs, S. (1954). *Enzymologia*, **16**, 193.
Charlwood, P. A. (1957). *Trans. Faraday Soc.* **53**, 871.
Dieu, H. A. (1957). *Biochem. J.* **66**, 19P.
Freudenberg, E. (1940). *Enzymologia*, **8**, 385.
Hanes, C. S., Hird, F. J. R. & Isherwood, F. A. (1950). *Nature, Lond.*, **166**, 288.
Harington, C. R. & Pitt-Rivers, R. V. (1944). *Biochem. J.* **38**, 417.
Harris, J. I. (1955). In *Methods of Biochemical Analysis*, vol. 2, p. 397. Ed. by Glick, D. New York: Interscience Publishers Inc.
Heirwegh, K. & Edman, P. (1957). *Biochim. biophys. Acta*, **24**, 219.
Herriott, R. M. (1938). *J. gen. Physiol.* **21**, 501.
Herriott, R. M. (1941). *J. gen. Physiol.* **24**, 325.
Herriott, R. M., Desreux, V. & Northrop, J. H. (1940). *J. gen. Physiol.* **23**, 439.
Levy, A. L. (1954). *Nature, Lond.*, **174**, 126.
Merten, R., Schramm, G., Grassmann, W. & Hannig, K. (1952). *Hoppe-Seyl. Z.* **289**, 173.
Moore, S. & Stein, W. H. (1948). *J. biol. Chem.* **176**, 367.
Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 907.
Northrop, J. H. (1930). *J. gen. Physiol.* **13**, 739.
Northrop, J. H. (1932). *J. gen. Physiol.* **15**, 29.
Northrop, J. H., Kunitz, M. & Herriott, R. M. (1948). *Crystalline Enzymes*, 2nd ed., p. 305. New York: Columbia University Press.
Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
Pope, C. G. & Stevens, M. (1951). *Brit. J. exp. Path.* **32**, 314.
Porath, J. (1956). *Biochim. biophys. Acta*, **22**, 151.
Porter, R. R. (1957). In *Methods in Enzymology*, vol. 4, p. 221. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Reindel, F. & Hoppe, W. (1954). *Chem. Ber.* **87**, 1103.
Richmond, V., Tang, J., Wolf, S., Trucco, R. E. & Caputto, R. (1958). *Biochim. biophys. Acta*, **29**, 453.
Ryle, A. P. (1958). *4th int. Congr. Biochem., Vienna*, 4–32.
Ryle, A. P., Sanger, F., Smith, L. F. & Kitai, R. (1955). *Biochem. J.* **60**, 541.
Sanger, F. (1949). *Biochem. J.* **44**, 126.
Sanger, F. & Tuppy, H. (1951). *Biochem. J.* **49**, 481.
Smithies, O. (1955). *Biochem. J.* **61**, 629.
Steinhardt, J. (1937). *K. danske vidensk. Selsk. Mat.-fys. Medd.* **14**, no. 11.
Taylor, W. H. (1959). *Biochem. J.* **71**, 73.
Troll, W. & Cannan, R. K. (1953). *J. biol. Chem.* **200**, 803.
Van Vunakis, H. & Herriott, R. M. (1957). *Biochim. biophys. Acta*, **23**, 600.

The Denaturation of Ovalbumin

CHANGES IN OPTICAL ROTATION, EXTINCTION AND VISCOSITY DURING SERIAL DENATURATION IN SOLUTIONS OF UREA

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Anfinsen & Redfield (1956) suggested an A-shaped structure for the native ovalbumin molecule, one leg of the A terminating in the carbohydrate moiety and the other bearing the *C*-terminal group. This structure was supported by a study of the increasing chemical reactivity of the ϵ-amino groups of lysine in ovalbumin during serial ethanol denaturation (Steven & Tristram, 1958). The authors postulated an internal link, which ruptured when the concentration of ethanol reached 50–55% (v/v). In earlier work, Maurer & Heidelberger (1951) observed a critical degree of deamination of ovalbumin above which the molecule appeared to uncoil, stressing the importance of amino groups in the maintenance of the native structure.

The present work is a study of the serial denaturation of ovalbumin in urea solutions complementary to the studies of Simpson & Kauzmann (1953), who observed that the rate of denaturation of a solution of ovalbumin at pH 7·6 increased rapidly in 7M-urea. It seemed probable that the extent of denaturation of ovalbumin as measured

by changes in physical properties, in urea solutions, would indicate a sudden increase at a critical urea concentration sufficient to rupture the postulated link. Schellman (1955) suggested there was a critical urea concentration for each protein at which the rate of denaturation was accelerated. If this is in fact true, the results presented here will not distinguish between the rupture of an internal link in ovalbumin and a non-specific action of urea on all proteins.

This critical urea concentration may be an indication of the energy required to rupture linkages within the protein essential for the maintenance of the native configuration. The concentration required might be expected to vary with the experimental conditions as well as with individual proteins.

METHODS

Materials. The (hen) ovalbumin was similar to that used by Steven & Tristram (1958). Urea (A.R.) as supplied by British Drug Houses Ltd. was used throughout.

Serial urea denaturation. The ovalbumin was dissolved and the solution brought to pH 11·6 with the addition of small quantities of 0·1N-NaOH. The pH was chosen in order to achieve a high protein concentration without danger of denaturation, which takes place above pH 12 (Crammer & Neuberger, 1943). The protein solution (10 ml.) was added to each of a series of flasks containing solid urea, each successive flask having 0·6 g. more urea than the previous one. In this way the initial urea strength was varied over the range 0–10M. The final strength, however, varies from 0 to 7·1M, owing to the increase in volume caused by the solution of urea. The initial urea strength will be used for convenience to describe the urea concentration, although both values are quoted in Fig. 1. The reaction mixtures were incubated at 20° for 18 hr., the pH in each adjusted to the initial value and the changes in physical properties were measured. These conditions will be referred to as the standard conditions in the following experiments.

RESULTS

Extinction. Crammer & Neuberger (1943) showed that the absorption maximum of ovalbumin in solution increased with pH both in wavelength and magnitude, the former increasing from 280 to 295 mμ during the conversion of the protein from the native into the denatured state. Glazer, McKenzie & Wake (1957) demonstrated a characteristic depression of the wavelength of maximum absorption during the urea denaturation of ovalbumin in acid solution from 280 to 277 mμ. The effect of pH on the absorption spectrum of denatured ovalbumin accounted for the differences observed by these two groups of authors.

In the present work the extinction was measured at 295 mμ; the extinction due to the presence of the urea was determined in a protein-free control experiment and this value was deducted from the observed extinction in the corresponding denaturation experiment. The changes in volume due to solution of urea were determined from the densities of the solutions, this necessarily introducing a correction factor for each concentration of urea to allow for the corresponding decrease in protein concentration.

The molar extinction coefficients (corrected for dilution and the absorption due to the urea) plotted against the initial urea molarity are presented in Fig. 1.

Iodination of tyrosine residues. Li (1942) studied the kinetics of the iodination of tyrosine and later (1945) employed the rates of iodination of these residues in pepsin and serum albumin to demonstrate increased chemical reactivity of tyrosine residues during urea denaturation.

In the present work samples of ovalbumin solution (1·5%, pH 11·6) were incubated under the standard conditions with urea. The final pH was adjusted to 5·7 by adding 1 ml. of M-acetate buffer of this pH containing 0·1M-KI. Each sample was treated with 2 ml. of aqueous 0·1N-I_2 solution and allowed to stand at room temperature for 30 min.

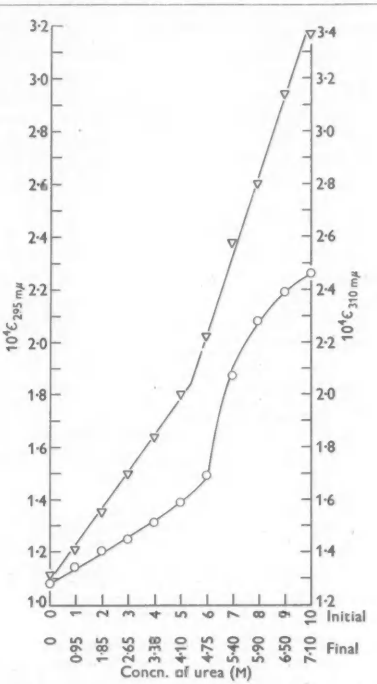

Fig. 1. Molar extinction coefficients of ovalbumin in urea solutions. ○, Tyrosine: $\epsilon_{295\,m\mu}$, pH 11·6; ▽, di-iodotyrosine: $\epsilon_{310\,m\mu}$, pH 12·0.

before the reaction was stopped by the addition of 2 ml. of 0·1N-$Na_2S_2O_3$. The precipitated protein was dissolved by adding 10 ml. of N-NaOH (bringing the final pH up to 12), the volume made up to 100 ml. and the extinction measured at 310 mμ. Crammer & Neuberger (1943) found the absorption maximum of di-iodotyrosine to be 315 mμ at pH 13. The absorption maximum of iodinated ovalbumin was 310 mμ at pH 12. The increase in the extinction, measured at 310 mμ, during urea denaturation should therefore be an indication of the degree of iodination of tyrosine residues. The increase in extinction cannot be used as a direct measure of iodination since the phenolic groups of tyrosine and di-iodotyrosine in the protein also absorb in the region 295–310 mμ at this pH.

A control experiment was set up in which the conditions were identical in all respects with those described above, except that the iodine and thiosulphate were mixed before addition to the protein solution. In this way no iodination could take place; the absorption was measured at 310 mμ over the complete range of urea concentration and no increase was observed. The control indicated that no correction need be made for the absorption caused by the reagents employed in the reaction measured at this wavelength.

The molar extinction coefficients at 310 mμ plotted against the initial urea molarity are presented in Fig. 1. The iodine reaction was carried out for 10, 20 and 30 min.; in each instance the increase in extinction for each solution remained constant.

Optical rotation. Rotations were measured at 20° with a Schmidt and Haensch (Berlin) polarimeter with the sodium D line. Serial urea denaturation of ovalbumin was carried out at 20° for 18 hr., under the following experimental conditions, before the rotations were observed: (*a*) standard conditions of 1·5% protein and pH 11·6; (*b*) standard conditions in the presence of 0·1M-sodium thioglycollate, which was added to the urea before mixing with the protein solution; (*c*) standard conditions but with the addition of 0·1M-Na_2SO_4 to the urea before mixing with the protein; (*d*) protein solutions made up in dilute HCl to pH 2·5.

The results obtained from these experiments are presented in Fig. 2 after allowing for the lowering of the protein concentration. The initial and final rotations, 30° and 100° respectively, are in good agreement with the values observed by Simpson & Kauzmann (1953). The slightly higher initial rotation observed in acid solution corresponds to a similar observation made by Schellman (1958*b*). Sulphate ions suppress the change in rotation over the whole range of urea concentration. The graphs indicate critical concentrations of urea at which the rotations become increasingly negative. The critical urea concentration is dependent on the experimental conditions, namely pH, the presence of a disulphide reducing agent or an inhibitor of denaturation such as sulphate ions.

Viscosity. Viscosities were measured at 20° with an Ostwald viscometer (79 sec. H_2O at 20°; Griffin and George Ltd., London). The small change in density between the control urea solutions and the urea solutions containing protein has been neglected. The small correction factor due to changes in refractive index in urea solution has also been ignored (Schellman, 1958*a*). The changes in viscosity observed during serial urea denaturation are presented in Fig. 3 as $(\Delta\eta/c)$ plotted against the initial urea concentration, after correcting for the dilution of the protein concentration. The results are presented in this way for the following reasons: If the reduced viscosity, $\left(\frac{[\eta_1-\eta_0]}{\eta_0}\right)\Big/c$, where c is the molar concentration, is employed at high concentrations of urea the change in viscosity, $\eta_1-\eta_0$, is zero, while the value of η_0 continues to increase; consequently the graph of the reduced viscosity versus the urea concentration may show

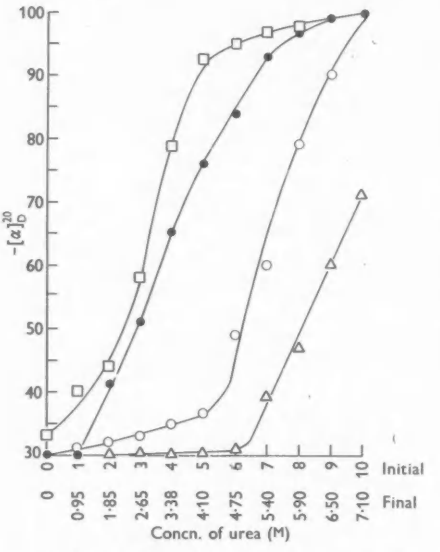

Fig. 2. Optical rotation of ovalbumin in urea solutions. (○), Standard conditions (see text) of 1·5% protein, pH 11·6; (●), standard conditions in the presence of 0·1M-sodium thioglycolate; (△), standard conditions in the presence of 0·1M-Na_2SO_4; (□), protein solutions in dilute HCl at pH 2·5.

a maximum before a decline. When $\Delta\eta/c$ is employed under identical conditions, the graph levels off at high concentrations of urea, at which the ovalbumin molecule appears to be fully uncoiled. The graph of $\Delta\eta/c$ against molarity of urea actually records the change in viscosity of the protein independently of any changes in the viscosity of the solvent.

Serial urea denaturation of the protein solution (1·5%) was carried out under conditions identical with those employed for the rotation experiments described above as (*a*), (*b*), (*c*) and (*d*). In each of these experiments the same type of graph was obtained as in the corresponding rotation experiment, the inflexion appearing at the same urea concentration. The increase in the final value of $\Delta\eta/c$ in alkaline pH as compared with the same conditions in the presence of thioglycollate or in acid pH is probably due to the cross-linking of the extended peptide chains through conversion of intramolecular disulphide bonds into intermolecular bonds (Frensdorff, Watson & Kauzmann, 1953). This type of cross-linking cannot take place at acid pH or in the presence of thioglycollate in alkaline solution.

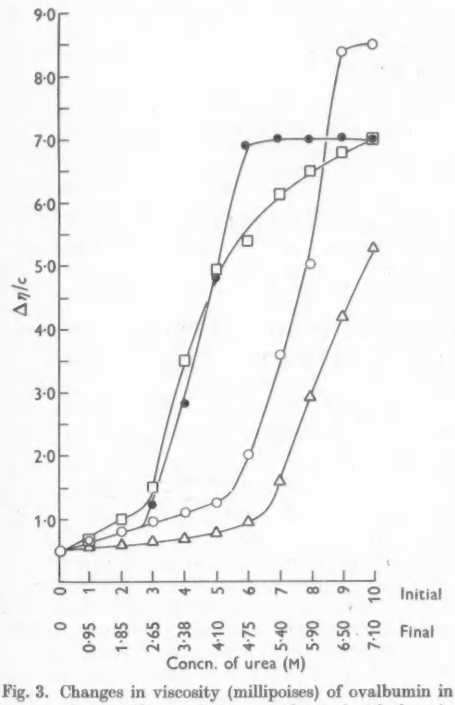

Fig. 3. Changes in viscosity (millipoises) of ovalbumin in urea solutions. The conditions are identical with those in Fig. 2, the symbols having the same meaning.

The results are compared in Table 1; the optical absorption studies on tyrosine and di-iodotyrosine are not included, since only conditions (*a*) were examined, although the inflexion occurred at an initial concentration of 5–6M-urea, as was observed in the corresponding rotation and viscosity changes.

DISCUSSION

The serial urea denaturation of ovalbumin, as measured by changes in certain physical properties, occurs in such a way as to suggest a critical urea concentration above which the configuration of the protein molecule is greatly altered. It is suggested that this critical urea concentration causes the rupture of some linkage which is essential for the maintenance of the native structure. Schellman (1955) suggested that the high electrostatic charge associated with a protein molecule at extremes of pH would produce repulsive forces within the chain and thus enhance the action of hydrogen-bond breakers. This would account for the difference observed in the critical urea strength of 7M at pH 7·6 (Simpson & Kauzmann, 1953) and 5–6M at pH 11·6 in the present work. The critical urea strength is lowered in acid and thioglycollate solutions indicating respectively the acid lability and possible structural importance of disulphide bonds in ovalbumin. The disulphide bonds in ovalbumin are not attacked by thioglycollate in the absence of urea, in contrast with the uncoiling

Table 1. *Variation of the critical urea concentration with experimental conditions*

In all experiments denaturation was continued for 18 hr. at 20°.

Experimental conditions	pH	Added reagent	Critical urea molarity (initial concn.) $[\alpha]_D^{20}$	Critical urea molarity (initial concn.) $(\Delta\eta/c)$
(*a*)	11·6	—	5–6	5–6
(*b*)	11·6	0·1M-Thioglycollate	1–2	2–3
(*c*)	11·6	0·1M-Na_2SO_4	6–7	6–7
(*d*)	2·5	—	2–3	2–3

action of thioglycollate on insulin (Schellman, 1958*c*).

The results suggest that a rapid uncoiling of the ovalbumin molecule occurs after a certain degree of denaturation has been exceeded, which is in agreement with the serial ethanol-denaturation study previously described.

SUMMARY

1. The degree of denaturation produced in ovalbumin by urea solutions under various conditions has been followed by physical means.

2. During denaturation optical rotation and viscosity show parallel changes and indicate that there is a critical urea concentration. This urea concentration is thought to be associated with the rupture of a linkage important for maintaining the configuration of the native protein.

3. Changes in extinction coefficient at 295 mμ and, after iodination, at 310 mμ also exhibit a similar dependence upon urea concentration.

REFERENCES

Anfinsen, C. B. & Redfield, R. R. (1956). *Advanc. Protein Chem.* **11**, 1.
Crammer, J. L. & Neuberger, A. (1943). *Biochem. J.* **37**, 302.
Frensdorff, H. K., Watson, M. T. & Kauzmann, W. (1953). *J. Amer. chem. Soc.* **75**, 5157.
Glazer, A. N., McKenzie, H. A. & Wake, R. G. (1957). *Nature, Lond.*, **180**, 1286.
Li, C. H. (1942). *J. Amer. chem. Soc.* **64**, 1147.
Li, C. H. (1945). *J. Amer. chem. Soc.* **67**, 1065.
Maurer, P. H. & Heidelberger, M. (1951). *J. Amer. chem. Soc.* **73**, 2070.
Schellman, J. A. (1955). *C.R. Lab. Carlsberg, Sér. chim.* **29**, 229.
Schellman, J. A. (1958*a*). *C.R. Lab. Carlsberg, Sér. chim.* **30**, 363, 439.
Schellman, J. A. (1958*b*). *C.R. Lab. Carlsberg, Sér. chim.* **30**, 429.
Schellman, J. A. (1958*c*). *C.R. Lab. Carlsberg, Sér. chim.* **30**, 415.
Simpson, R. B. & Kauzmann, K. (1953). *J. Amer. chem. Soc.* **75**, 5139.
Steven, F. S. & Tristram, G. R. (1958). *Biochem. J.* **70**, 179.

Kinetics and Equilibria in Systems Containing Haem, Carbon Monoxide and Pyridine

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The object of the work described here was to compare the kinetic behaviour of haem compounds of varying degrees of complexity. A single reaction, combination with carbon monoxide, was studied to determine if the rate of combination was variable and the structural factors upon which any variation depended. Three groups of haem compounds were chosen: free haem, the haemochromogens and the haemoproteins. Myoglobin was taken as representative of the last-named group, being formally similar to the simpler haem compounds in having only one haem group per molecule. It also combines reversibly with carbon monoxide, and is very similar in many of its properties to haemoglobin. For the haemochromogen group the compounds with pyridine were chosen, since pyridine has been very widely used and more information was available than for other possible ligands. The effect of changing the side chains on the porphyrin nucleus has also been examined, and mesohaem and deuterohaem and their derivatives have been prepared in addition to protohaem. The main problem therefore was to determine the rates of combination of carbon monoxide with three closely related haems, three pyridine haemochromogens and three myoglobins (nine reactions in all), the whole representing a gradual increase in the complexity of the haem-conjugated molecule.

The results with myoglobin conveniently stand alone and are dealt with in the next paper (Smith & Gibson, 1959). The present paper is divided into two parts: the first deals with experiments designed to establish the nature of the materials being studied, and the conclusions drawn about their structure; the second gives the results of the kinetic experiments.

It has been generally accepted that the iron atom in a haem molecule is able to combine with only two other groups, one on each side of the haem ring (cf. Lemberg & Legge, 1949). Although the concept of facultative hepta-co-ordination could resolve certain experimental and theoretical difficulties, its use seems to lead, in the end, to even greater perplexities than exist at present. In this work it

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has been assumed that only a sixfold linkage of the iron need be considered, and this is sufficient to explain the observed results and to relate them to previous findings.

MATERIALS AND METHODS

Haemins. Protohaemin was prepared by Drabkin & Austin's (1935) modification of the method of Schalfejeff (1885). A sample of haemin from British Drug Houses Ltd. containing 8·46% of Fe was also used. Mesohaemin was prepared from protohaemin with the reagents of Fischer & Gibian (1941), but no reaction took place when the mixture was allowed to stand for 24 hr. at room temperature. Satisfactory yields were obtained when the reaction mixture was refluxed gently for 15–20 min. Deuterohaemin was prepared from protohaemin by the method of Treibs (1933) as described by Walter (1952). All the samples of haemin were crystallized from acetic acid by the method of Hogness, Zscheile, Sidwell & Barron (1937).

Sodium dithionite. A sample of Manox dithionite was obtained from Holdman and Harden, Miles Platting, Manchester, 1.

Spectrophotometry. The concentration of solutions was determined with a Unicam SP. 600 spectrophotometer. Drabkin (1942) found that the maximum extinction coefficients for several derivatives of meso- and coprohaem had the same numerical values as those for the corresponding derivatives of protohaem. It therefore seemed reasonable to use the value of Hogness *et al.* (1937) for the extinction coefficient of protohaemin, $\epsilon^{\text{mM}}_{490\,\text{m}\mu} = 6{\cdot}55$ for all three haematins. Readings were taken at 482 mμ for meso- and deutero-haematin, however. All readings were taken in a 1 cm. cell unless otherwise stated.

The millimolar extinction coefficients are defined as $\epsilon = E/cl$, where C is millimolarity of the haem solution, l is optical depth and E (observed extinction) is $\log I_0/I$, where I_0 and I are the intensities of the incident and transmitted light respectively.

Preparation of solutions. As neither haematin nor dithionite is appreciably soluble in glycol alone, the following standard procedure was used throughout. Stock solutions of haematin in 0·02 N-NaOH were made up as required in sufficient strength to give the required extinction after dilution. To 0·5 ml. of a stock solution of haematin (0·15 mM), 1 ml. of 2·5% (w/v) $Na_2S_2O_4$ in 0·02 N-NaOH was added and the mixture diluted to 10 ml. with 0·02 N-NaOH. This gave a haem solution suitable for spectrophotometric or kinetic experiments in the Soret region with a 1 cm. optical cell. If pyridine was to be introduced into the system this was done by adding the necessary small volume of a stock solution in water before making up to 10 ml. with 0·02 N-NaOH. Where ethylene glycol was to be used, the haem solution was made up to 10 ml. with ethylene glycol instead of NaOH. There was always at least 70% (v/v) of glycol and usually 80–90%. Some preliminary experiments suggested that about 60% (v/v) was required to produce the full effect on the spectrum.

Carbon monoxide–haem dilution test. Carbon monoxide–haem was examined over the range 0·075–65 μM by recording the change in extinction at 406·5 mμ, the Soret maximum of the substance. Various depths of cell from 0·1 to 4·0 cm. were employed to obtain measurable extinctions at all concentrations. These were all subsequently converted into the corresponding values for a 1 cm. cell.

Carbon monoxide–haem dissociation curve. The methods used to establish the curve consisted of (i) direct equilibration of haem solutions with varying partial pressures of CO, and (ii) mixing aqueous solutions of known concentrations of haem and CO in a closed system and observing the change in extinction. In both the extinction at 406·5 mμ was recorded, the fractional saturation then being given by the increase of extinction over that of the free haem, divided by the increase on forming fully saturated CO–haem from free haem.

Flash-photolysis kinetic measurements were carried out with the apparatus and procedure of Gibson (1959).

Photochemical absorption spectrum. The construction of the flash-photolysis apparatus used in the kinetic work made it possible to calculate the change in extinction of the solution being examined, at the moment of flashing. If the initial spectrum was known, then the absolute spectrum immediately after flashing could be calculated, as well as the difference spectrum. The former corresponds to the 'photochemical absorption spectrum' of Warburg & Negelein (1929) and Lemberg & Legge (1949), but is now obtained by direct measurement.

RESULTS

Structure of haem and carbon monoxide–haem

Spectrophotometric observations. Much of the available information about the simpler haem compounds has come from spectrophotometric work. So far as haem and its derivatives are concerned, it seems (Shack & Clark, 1947) that a distinction can be drawn between monomeric and dimeric forms in aqueous solution on the basis of their absorption spectra. These criteria appear to be applicable also to haem derivatives in non-aqueous solvents. The general finding is that the monomer has a much sharper spectrum than that of the dimer (which is usually markedly rounded-off and often shifted to a longer wavelength than that of the monomer), and that the monomer has a much greater extinction at the absorption maximum.

Comparison of the absorption spectra of free haem and of CO–haem in aqueous solution (Figs. 1 and 2) shows that on this basis the former is dimeric and the latter monomeric. Fig. 1 shows the dramatic difference between the absorption spectra of free haem in aqueous solution and in glycol. On the premise that aqueous haem is dimeric, the considerable increase in extinction and shift in position of the Soret band, together with the form of the new curve, lead to the conclusion that the solution in glycol contains monomeric haem.

Use of ethylene glycol. It is generally recognized that many substances which occur as polymers in aqueous solution tend to split up into lower polymers and eventually monomers in organic solvents

(Rabinowitch & Epstein, 1941; Lewis, Magel & Lipkin, 1942; Lewis, Goldschmid, Magel & Bigeleisen, 1943). The use of ethylene glycol in this respect is cited in the paper by Lewis *et al.* (1943), and it has recently been used for the same purpose in this Department (Weber & Teale, 1958). The findings could not be fully confirmed with other solvents as several common organic solvents, including ethanol and acetone, were found to be unsuitable because of the formation of a white precipitate, presumably as a result of reaction with the dithionite (a sulphurous smell was usually noticeable). The general effect of these solvents, however, appeared to be similar to that of glycol.

With CO–haem (Fig. 2) there is only a slight increase in absorption at the maximum and no change in the form of the curve. It is not clear whether the increase is due directly to the change in the solvent, or whether it is due to the presence of small amounts of free (dimeric) haem in aqueous solutions of CO–haem. Observations made in this Department by Dr G. Weber (personal communication), on the effect of different solvents on the spectra of various aromatic nitrogenous compounds, suggest that the former is very probable.

Dilution test. When the observed extinctions at various dilutions were converted into the corresponding values for 10 cm. cell, the plot of extinction against concentration gave a straight line passing through the origin. Although confirming that the material obeyed Beer's law down to very low concentrations, this result could not by itself be taken as evidence that the CO–haem was monomeric. Reproducible results could not be obtained in similar experiments with free haem.

Dissociation curve. Some time was spent on establishing the dissociation curve of CO–haem, in view of the information which this might give about the system. It was found difficult to obtain consistent results, partly owing to the high affinity of haem for CO, which tended to render observations difficult by the methods available, and partly because of unexplained differences between the earlier and subsequent results. In each case several different solutions of haem were used. The differences are at present tentatively attributed to variations in the state of aggregation of the haem. The earlier results indicated that in 0·02N-NaOH at room temperature (about 20°) the haem was 50% saturated at an equilibrium value of 0·75 mm. Hg CO or 1·0μM. Thirty-three points obtained over the whole range of the dissociation curve in six experiments were analysed by the method of least squares, giving K 0·92 × 10^6 (K = [CO–H]/[CO] [H] concentrations in moles, where H is the haem molecule and n = 1·17). The results were compatible with a value of n = 1. (In the Hill equation, the value n gives a measure of the degree of

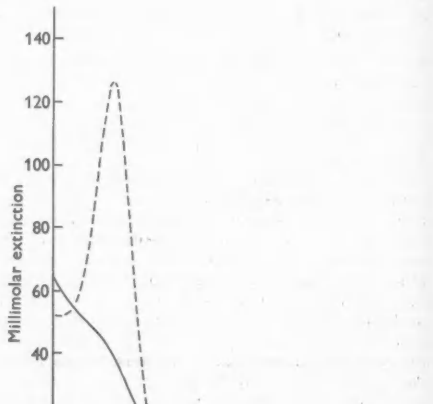

Fig. 1. Absorption spectra in the Soret and visible regions of alkaline protohaem, in 0·02N-NaOH (—) and in 80% (v/v) ethylene glycol (– – –). [Haem] 7μM; light path 1 cm. pH of aqueous solution 11·5.

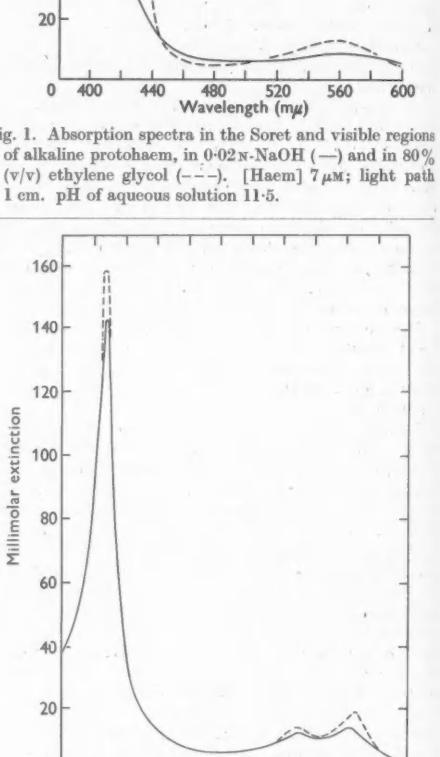

Fig. 2. Absorption spectra in the Soret and visible regions of alkaline CO–protohaem, in 0·02N-NaOH (—) and in 80% (v/v) ethylene glycol (- · - · -). [Haem] 7μM; light path 1 cm.

interaction of the haems: $n = 1$ means either that the substance is monomeric or that it is polymeric but with negligible interaction.) Later experiments gave 50% saturation in the range 3·5–4·5 μM-CO (mean about 3 mm.Hg CO with $K = 0{\cdot}33 \times 10^6$). Both sets of results were obtained under apparently identical conditions.

Since this section of the work was completed, Wang, Nakahara & Fleischer (1958) have presented what appears to be the first published set of data for the dissociation of CO–haem. Their figures indicate a half-saturation value of about 3·1 μM (2·4 mm.Hg CO), at approximately 24·6°. The curve plotted from their figures is a rectangular hyperbola, passing through the origin, from which n may be calculated to equal 1·0. These figures, which agree very well with the second set of those given above, are apparently based upon a single set of observations, and there is no mention that variations can occur.

Photochemical absorption spectrum. The photochemical absorption spectrum of CO–haem solutions showed peaks at 406 mμ and at 420 mμ. The latter corresponds in position to the absorption maximum of haem in glycol. The changes are not yet fully explained since it appeared that even in glycol an appreciable fraction of the CO–haem was not dissociated; yet increasing the intensity of the flash did not give the expected increase in photolysis. It is presumed that the reactive portion yields monomeric haem even in aqueous solution, and that this then recombines with CO, possibly with dimeric haem as an intermediate.

Structure of pyridine and carbon monoxide–pyridine haemochromogens

Changes in extinction at a single wavelength. By varying the concentration of pyridine in equilibrium with a fixed amount of haem it may be shown that, at the concentration of haem used, there must be more than one compound formed. The changes were most easily followed in the Soret region, and in order to use 1 cm. cells the haem solutions were made up to between 5 and 10 μM. (No variation in the results was noted which could be ascribed to differences in concentration over this range.) Although the conclusions drawn from experiments with this dilute haem have been tentatively assumed to apply also to somewhat stronger solutions (up to at least 0·1 mM), only a few observations have so far been made with the stronger solutions. The general findings presented in this and other sections of this paper therefore refer directly to the more dilute solutions of haem, and some care must be taken in extending them to stronger solutions.

To show the changes in extinction with increase in pyridine concentration, the composite figure shown in Fig. 3 was built up from a number of single observations with varying amounts of pyridine. It was found that as the concentration of pyridine was increased, the extinction observed at a single wavelength increased in a regular manner. At first there was a small increase, approaching completion at about mM-pyridine. This change was best observed at 410 mμ (see next section); subsequent changes were best observed at 420 mμ. As the pyridine concentration was raised more, there was a further and larger increase in absorption, which reached a maximum at about 60 mM-pyridine. One further increase in extinction took place on raising the pyridine concentration, and this was maximal at about 10M-pyridine. No further increase occurred, even in 80% (v/v) pyridine. The last two stages are quite clear-cut and easily reproducible, and although replicate experiments did not necessarily agree exactly, their general form was very similar. The initial curve was not always easy to demonstrate, partly owing to the small change in extinction, and possibly also owing to differences in the state of aggregation of the haem.

Dr Philip George and Mr Alan Adler of the John Harrison Laboratory, University of Pennsylvania, have confirmed (personal communication) that the spectrophotometric titration of pyridine against haem gives a stepped curve of the type described. Their experiments were done within 3 hr. of making up the haemin solution and at pH 7·0. As in the work reported above, they found observations of the initial stages to be the most difficult. In a

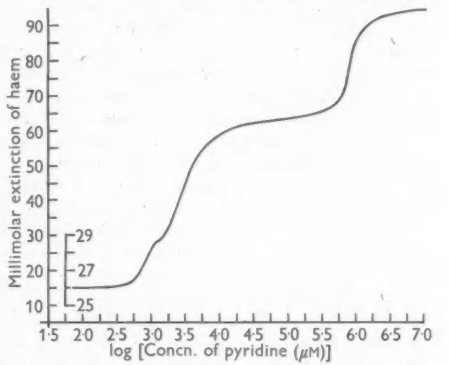

Fig. 3. Change in extinction at a single wavelength on adding pyridine to alkaline protohaem (data from a single experiment). Extinction was observed at 420 mμ. [Haem] 12 μM throughout; light path 1 cm. Pyridine concentration in final solution varied from 0 to 10M. Maximum millimolar extinction at 418 mμ was more generally found to be about 130. Inset scale applies to first step only, i.e. to 3·25 units on abscissa.

typical experiment maximum formation of the last two compounds occurred at about 70 mM- and 2·5M-pyridine. The millimolar extinction of haem at 418 mμ at these points was 67·0 and 188. The latter value is considerably higher than was found in the present work (see Figs. 3 and 4). Adler & George report that, in general, higher values for the extinction of pyridine haemochromogen were found than are given in the literature, and that this may possibly be correlated with the age of the haem solution employed. On the other hand, however, experiments involving the oxidized form from the same stock solution gave extinction coefficients similar to, or slightly lower than, those previously reported. Experimental conditions used by these workers were: haematin, 8μM in 0·2M-phosphate buffer, pH 7·0; reduction by solid dithionite; whole spectra in the Soret region were obtained with a Beckman Recording Spectrophotometer with *l* 1 cm.

The curve shown in Fig. 3 demonstrates that the stepwise addition of pyridine forms no less than three compounds with haem. The changes were most easily observed in the Soret region owing, as will be shown in the next section, to the wide separation of the absorption maxima of the three forms in this region. They can also be observed, but with more difficulty, in the visible region, where all three forms show the typical two-banded haemochromogen spectrum. Investigation of the visible absorption spectra has not yet been detailed enough to show with certainty whether or not a shift in wavelength accompanies the increase in extinction. If there is a shift it is only of the order of 2 mμ. Shack & Clark (1947), using 0·1 mM-haem, reported that 'on further addition of pyridine [to haem] the peak near 560 mμ progressively shifted to lower wavelengths'. The extent of the shift was not stated. In the present work the absorption maximum of (monomeric) pyridine haemochromogen was found to be at 555 mμ, as measured in the Unicam SP. 600 spectrophotometer [but cf. Drabkin's (1942) value of 558 mμ]. The Soret extinction of free haem in glycol, at 420 mμ, so closely resembles the peak of pyridine haemochromogen in glycol in position and extinction coefficient that observations on the change from one to the other are difficult to make. In the visible region with glycol only one stage has been observed, corresponding to the last stage in NaOH; the change is maximal at 0·1M-pyridine. A few experiments with the meso- and deuterohaem were less satisfactory, since the absorption bands of the different compounds are closer together than with protohaem, and there is overlapping of absorption bands in the reactions with pyridine. There is, however, no reason to suppose that the properties of meso- and deutero-haem in these systems are different from those of protohaem (cf. Walter, 1952).

Absorption spectra of pyridine haemochromogen with varied pyridine concentration. Examination of the absorption spectra in the Soret region during the determination of the pyridine dissociation curves shows three distinct types of spectrum, which merge to give composite spectra at intermediate concentrations of pyridine. Fig. 4 shows the observed spectra in the Soret region of free haem, and of the solution at stages corresponding to the maximum formation of each intermediate compound.

Further spectrophotometric observations in the Soret region are set out in Figs. 5–8. In the visible region the usual maximal formation of each of the three pyridine haemochromogen compounds and CO–haemochromogen spectra have been observed, but the changes have not yet been followed so closely as in the Soret region.

In the presence of CO the spectral changes are simpler (all experiments on the effect of CO were carried out by equilibrating the system with the gas at a pressure of 1 atmosphere). The CO–haem peak at 406 mμ (Fig. 5) shifted to about 410 mμ and became more rounded (Fig. 6). With 50–100 mM-pyridine the maximum extinction was

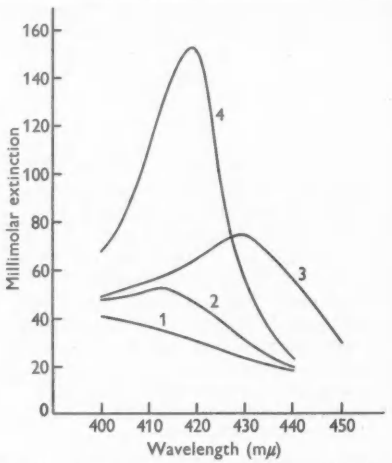

Fig. 4. Absorption spectra of free alkaline protohaem and haem–pyridine compounds in the Soret region, in 0·02N-NaOH. pH of solution 11·5. The three spectra in the presence of pyridine represent the observed spectrum at maximum formation of each intermediate haem-pyridine compound (cf. Fig. 3). Pyridine concentration in the observed solution: curve 1, 0 (free haem); curve 2, 1–2 mM; curve 3, 50–100 mM; curve 4, 10–13M. Maximum millimolar extinction at 418 mμ was more generally found to be about 130. [Haem] 12μM; light path 1 cm.

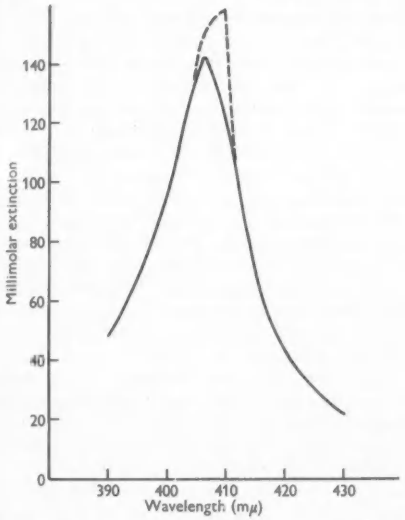

Fig. 5. Absorption spectra in the Soret region of the γ-band of alkaline CO–protohaem, in 0·02 N-NaOH (—) and in 80% (v/v) ethylene glycol (– – –). (No pyridine was present.) [Haem] 7 μM; light path 1 cm.

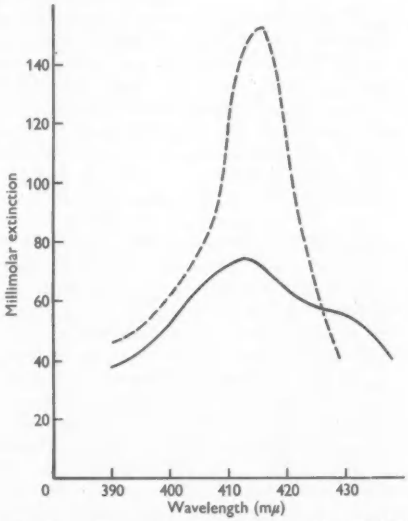

Fig. 7. Absorption spectra in the Soret region of alkaline CO–protohaem with 50–100 mM-pyridine, in 0·02 N-NaOH (—) and in 80% (v/v) ethylene glycol (– – –). The spectrum in aqueous solution demonstrates incomplete conversion into the monomeric CO–haemochromogen (peak at 418 mμ), the shoulder at about 430 mμ being due to the presence of dimeric dipyridine haemochromogen (see Fig. 4). [Haem] 7 μM; light path 1 cm.

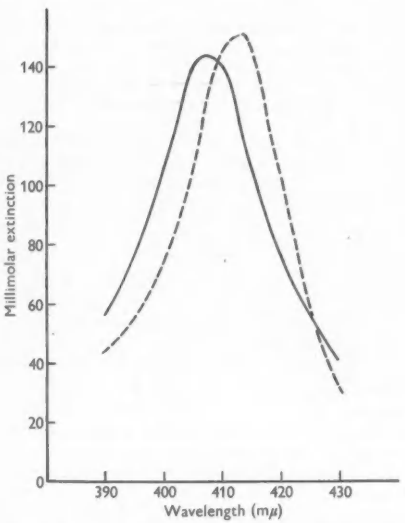

Fig. 6. Absorption spectra in the Soret region of alkaline CO–protohaem with 1–2 mM-pyridine, in 0·02 N-NaOH (—) and in 80% (v/v) ethylene glycol (– – –). [Haem] 7 μM; light path 1 cm.

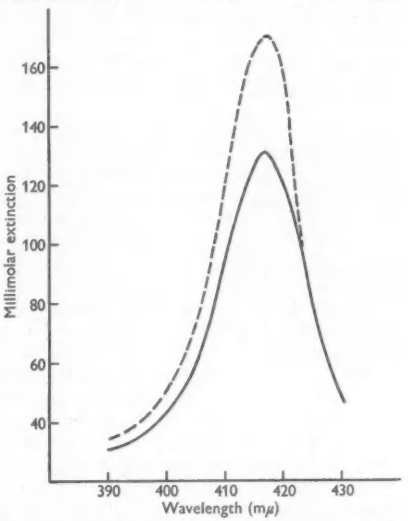

Fig. 8. Absorption spectra in the Soret region of alkaline CO–protohaem with 10–13 M-pyridine, in 0·02 N-NaOH (—) and in 80% (v/v) ethylene glycol (– – –). [Haem] 7 μM; light path 1 cm.

about 65% of the original value. There was little further change until the pyridine concentration was raised above 0·4M, when a sharper peak emerged at 418 mμ (Fig. 8). It was found to be essential to add pyridine to CO–haem thoroughly saturated with CO, not to bubble CO through pyridine haemochromogen solution. In the latter case the peak at 430 mμ, formed in the intermediate range of pyridine concentration, remained even after passing CO (Fig. 7). It was necessary to ensure thorough saturation with CO because this same peak could appear even when the pyridine was added last, presumably through combination of pyridine with free haem.

Ethylene glycol solutions were again used to supplement the observations with aqueous solutions. In the absence of CO there was only a single peak at 418 mμ, higher and sharper than corresponding peaks in NaOH, at all concentrations of pyridine. There appeared to be a slight increase in the height of the peak with increasing pyridine concentration. At the highest pyridine concentration the peak in glycol resembled the corresponding one in NaOH very closely, but gave a slightly higher extinction. In the presence of CO exactly the same behaviour was observed, but the extinctions were in all cases greater than those in absence of CO and did not vary very much with change in pyridine concentration.

Photochemical absorption spectrum. Photochemical absorption spectra were taken at 1·0 mM- and 2·3M-pyridine. In each case the spectrum showed a maximum at 415–420 mμ. In the first case this represented an increase in extinction over this range; in the second it represented a decrease.

Kinetic results

Carbon monoxide–haem. Carbon monoxide–haem is commonly regarded (cf. Lemberg & Legge, 1949) as being photosensitive, yet the first experiments appeared to show that no reaction was taking place. Examination of the literature showed that the published evidence for photosensitivity derived from the observations of Warburg & Negelein (1929) and Krebs (1929). These workers described manometric experiments in which haem catalysed the oxidation of cysteine to cystine. The presence of cysteine at once rendered their interpretations suspect since the work was done before it was shown (Keilin, 1950) that a number of amino acids can form haemochromogens, and the authors did not appear to have ruled out this possibility. However, it has not proved possible to obtain such a haemochromogen under the conditions employed in the present work, nor is there any sign of the formation of a mixed CO–cysteine compound with the haem. Presumably a similar situation existed in the experiments cited, where the haem was dissolved in phosphate and borate buffers at pH 6·8 and 10·3.

It was subsequently possible to demonstrate the photolysis of the CO–haem, the reason that this had not been observed earlier being due to the rapidity of the recombination reaction. This was found to be some 40 or more times faster than the CO–myoglobin reactions already studied (Smith & Gibson, 1959), and could only be observed in the existing apparatus by reducing the partial pressure of CO to not more than 10–20 mm. Hg.

In view of the technical difficulties in measuring these very fast reaction rates, which are among the fastest reactions known involving biochemical substances, the results in Table 1 must be regarded as approximate only.

Carbon monoxide–haemochromogens. So far, no evidence has been found that any of the pyridine haemochromogens is photosensitive in the absence of CO. In experiments in which increasing amounts of pyridine were added to CO–haem it was found that the very rapid CO–haem reaction gradually became biphasic, eventually giving a much slower, monophasic reaction. At still higher pyridine concentration this too became biphasic and finally there was a very slow, apparently monophasic reaction at M or greater concentrations of pyridine.

Table 1. *Rate of combination of proto-, meso- and deutero-haem with carbon monoxide*

Concentration of the haem was 7 μM, of CO ~ 20 μM, temperature 20°. Observations were made over the range 410–430 mμ, usually at 430 mμ. Solutions with glycol contained 80% (v/v): in all cases the concentration of $Na_2S_2O_4$ was 0·25% (w/v). Flash-photolysis energy 400 J; light path 1 cm.

Haem	Solvent	10^{-7} × Velocity constant (M^{-1} sec.$^{-1}$)
Proto-	0·02N-NaOH	1·6
	Glycol	1·4
Meso-	0·02N-NaOH	8·7
	Glycol	5·1
Deutero-	0·02N-NaOH	8·4
	Glycol	—

Table 2. *Rate of combination of monopyridine haemochromogens with carbon monoxide*

Haem (7 μM) was dissolved in 0·02N-NaOH and reduced with dithionite as described in the section on Methods. [CO] 140 μM. Temperature 20°: Flash-photolysis energy 100 J; reaction was followed by the change in absorption at 420 mμ.

Haem	10^2 × Pyridine concentration (M)	10^{-6} × Velocity constant (M^{-1} sec.$^{-1}$)
Proto-	4·0	0·42
Meso-	6·5	1·04
Deutero-	10·5	1·30

These findings may be explained by supposing that the first biphasic reaction is due to the presence in the same solution of CO–haem and CO–haemochromogen (both monomeric). The reaction with the haemochromogen is much slower than the reaction with haem, and only when sufficient pyridine has been added to convert all of the haem into haemochromogen does the reaction become monophasic. This result can only be observed over a small range of pyridine concentration, further increase in the amount of pyridine causing the tracing to become biphasic again. The appearance of a second component in the reaction at this point is not believed to be due to a new reacting species but to result from competition between pyridine and CO for a place on the haem molecule. The relative affinities appear to be such that with millimolar CO (1 atm. partial pressure) a fairly high proportion of the haem is in combination with CO even at the highest pyridine concentrations. This equilibrium state is upset by flash photolysis and with high pyridine concentrations dipyridine haemochromogen, rather than monopyridine–water–haemochromogen, is formed immediately after flashing. Carbon monoxide is unable to combine directly with dipyridine haemochromogen and the rate of the observed reaction is no longer determined by the combination velocity constant for the reaction: monopyridine haemochromogen + CO → CO–pyridine haemochromogen, but by the dissociation velocity constant of the reaction dipyridine haemochromogen → monopyridine haemochromogen + pyridine. This replacement reaction, which has a half-time of several seconds, forms the slowest component of the reaction at high pyridine concentrations.

In practice it was found to be difficult to establish the precise values for the reaction: monopyridine haem + CO → CO–pyridine haemochromogen, owing to the tendency for the results to be influenced either by the CO + haem reaction (fast) or by the replacement reaction (slow). The reaction could only be observed over a relatively small range of pyridine concentrations and the values in Table 2 are taken from the results of a single experiment carried out after the explanation given above had been worked out, but they are in general agreement with between three and six experiments for each haem which had been previously made under less fully suitable conditions.

DISCUSSION

The work of Shack & Clark (1947) and of Walter (1952) has established that in aqueous solution free haem is present in the form of dimeric units aggregated into micelles. These authors did not, however, investigate the influence of carbon monoxide upon such systems. Carbon monoxide–haem was represented by Hill (1926) as CO–H–X, 'where X is either another molecule of the complex or a molecule of water', the symbol H representing the haem nucleus. If the former possibility were correct, then the formula would in fact be CO–H-H–CO, i.e. two carbon monoxide molecules combined with dimeric haem. Although this possibility does not seem to have been definitely excluded by later workers (cf. Keilin, 1949*a*, *b*) it has been generally assumed that carbon monoxide–haem is monomeric. Combination of pyridine with haem was shown by Hill (1926, 1929) to lead to the formation of a monomeric haemochromogen consisting of one haem nucleus combined with two pyridine molecules. The later work of Shack & Clark (1947) and of Walter (1952) has suggested that the formation of the monomeric haemochromogen from dimeric haem may proceed stepwise, although no definite evidence has been presented that this is so. With the combination of haematin with pyridine, these workers have shown that there is in fact a pH-dependent transition from dimer to monomer, each form being combined with two molecules of the base. The effect of carbon monoxide on haemochromogen systems has not yet been studied from the point of view of structure, and the available evidence has always been interpreted as due to the reaction of carbon monoxide with a monomeric haemochromogen. The final product is known (Hill, 1926) to contain iron, carbon monoxide and pyridine in equal proportions and can only be monomeric.

The general findings of the present spectrophotometric work have served to confirm the tentative division of haem compounds into dimers and monomers, postulated by Shack & Clark (1947) on the basis of the observed absorption spectra, as well as the general shift of the absorption bands of compounds of meso- and deutero-haem towards the blue, as compared with those of protohaem, already demonstrated by Drabkin (1942). The finding that solutions of haem reduced with dithionite may be readily and safely prepared in ethylene glycol has made it possible to provide direct spectrophotometric evidence for such a classification, and has proved a useful tool for the investigation of complex haem systems.

Haem and carbon monoxide–haem. The experiments with haem and carbon monoxide–haem support the belief of earlier workers that these substances in aqueous solution are normally dimeric and monomeric respectively. A change from dimeric haem to a monomeric form can be demonstrated by changing the solvent (cf. Maehly & Åkeson, 1958). With carbon monoxide–haem there is very little spectral difference in the two solvents, and the findings in the dilution test and

dissociation curve also support the view that the substance is normally monomeric. The photochemical absorption spectrum serves as a link between carbon monoxide–haem and free haem, by suggesting that monomeric haem is formed from carbon monoxide–haem even in aqueous solution. This change may be represented by the equation:

$$CO\text{–}H\text{–}H_2O + H_2O \rightleftharpoons H_2O\text{–}H\text{–}H_2O + CO$$

where H represents the haem nucleus. (Presumably the monomeric haem combines immediately with a water molecule, not remaining with a free valency.) In view of the general agreement with Hill's (1926) finding that the ratio of iron to carbon monoxide was 1:1 in carbon monoxide–haem, indicating the presence of monomeric haem, it seems likely that there is a straightforward conversion into monomeric carbon monoxide–haem at all haem concentrations.

Pyridine haemochromogen and carbon monoxide–pyridine–haemochromogen. The dissociation curves and absorption spectra of pyridine haemochromogen systems have shown that the addition of pyridine to haem can take place in a stepwise manner. Since the starting material (haem) is dimeric, only two dimeric and two monomeric haem–pyridine compounds are theoretically possible. In ethylene glycol only the two monomeric forms can occur.

Aqueous

H_2O-H-H-H_2O
↓
P-H-H-H_2O (III)
↓
P-H-H-P (II)
↓
P-H-P (I)

Glycol

EG-H-EG
↓
P-H-P (I)

H, Haem; P, Pyridine; EG, Ethylene glycol.

Scheme 1

Experiment showed that in aqueous solution there were three compounds, and in glycol only one. Scheme 1 is therefore proposed to account for the various observations. It is well known (Hill, 1929) that pyridine haemochromogen contains two pyridine molecules per iron atom. Shack & Clark (1947) and Walter (1952) agree that this form is monomeric. This makes it quite clear that the final pyridine–haem compound (I) has the formula indicated. In order to account for its formation in the number of steps given by the dissociation curves, it is necessary to postulate that the first pyridine molecule adds to the haem to give (III), which by addition of a second pyridine molecule passes to (II). This sequence is preferred to the alternative formulation of the monomeric monopyridine compound as the intermediate, on the following grounds: (i) the intermediate spectrum appeared to be that of a dimeric, rather than a monomeric, molecule, and has not been observed in glycol solutions, where only monomeric haem compounds have so far been identified; (ii) it did not appear in solutions of carbon monoxide–haem to which pyridine was added. On the other hand, its spectrum persisted as a secondary peak (see Fig. 7) if carbon monoxide was passed into solutions of (II). These qualitative observations suggest that (II) is not easily formed from the monomeric carbon monoxide–haem, nor does it readily give rise to this compound. Moreover, the dissociation curve of the pyridine compound in glycol appeared to be indicative of a change from free haem directly to haemochromogen I. The evidence therefore favoured the supposition that a monomeric monopyridine haemochromogen was not a normal intermediate either in aqueous solutions or in ethylene glycol. It is presumably formed on the photolysis of carbon monoxide–haemochromogen, and is tentatively assumed to have an absorption spectrum very similar to that of the dipyridine compound.

CO–H–H_2O
↓
CO–H–P
|
↓
P–H–P (I)

Scheme 2

The explanation given above can be correlated with observations on systems containing carbon monoxide, as is shown in scheme 2, which is applicable both to aqueous systems and those containing ethylene glycol. Here, the only possibility in either solvent is for the carbon monoxide–haem to form the carbon monoxide–haemochromogen, and this is in accordance with spectrophotometric observation. It is well known that pyridine can compete with carbon monoxide for a place on the haem molecule. Under certain conditions therefore the system may pass over wholly or partially to haemochromogen I. This possibility is believed to explain the kinetic results at high pyridine concentrations, as discussed above.

Previously published work has not been found which can be directly correlated with these findings, other than the suggestions of Shack & Clark (1947) and of Walter (1952) that association might be stepwise. Most workers have either used strong haem solutions, or else have investigated systems containing oxidized haem only. The closest analogy appears to be with the haem–cyanide system, where stepwise association undoubtedly occurs (Hogness *et al.* 1937; Keilin, 1949*a*). However, there does not seem to be

any possibility that dimeric compounds exist in the cyanide system, and it is actually more directly comparable with the carbon monoxide–haem system than with the haem–pyridine system, with the distinction that a compound of haem with two carbon monoxide molecules has not yet been identified. The conclusions drawn about the structure of various components of the systems examined are in good agreement with the findings of other workers. Although it remains to be seen if these conclusions are fully applicable to the stronger solutions used in potentiometric work, for instance, there is no doubt that they have to be taken into account when working with haem solutions up to 20–30 mM. In the common case of the estimation of haem as the pyridine haemochromogen the conventional use of 10–20% (v/v) pyridine (1·2–2·4M) has doubtless obviated anomalous results, as well as delaying the recognition of the changes in the haem–pyridine system.

As no kinetic data have hitherto been available for the combination of carbon monoxide with the simple haem compounds investigated, it is not possible to compare the results with those of previous workers. Nevertheless, they seem to be on the whole so clear-cut that definite conclusions may be drawn from them.

Comparison of the results given in Table 1 for the free haems with those in Table 2 shows a reduction in the combination rate on passing from free haem to the pyridine chromogen. This reduction is so large as to suggest that something more fundamental than a mere steric effect is operating. In any case, the results of the present investigations have demonstrated that the respective substances are both monomeric and, although it is not possible altogether to rule out the possibility that there is loose aggregation of the molecules through the side chains of the porphyrin nucleus, there is no reason to suppose that the photochemical reaction or the recombination reaction are affected by this.

Apart from steric and concentration effects, the velocity of a chemical reaction depends upon the ease with which bonds may be broken and formed. Where the co-ordination number of the iron appears to be less than six, as in the products of the photochemical reaction, it has been assumed that water fills the remaining place. In the reactions to be considered, the bond between the haematin iron and a water molecule must presumably be broken before the carbon monoxide compound can be reformed. Since this applies in all the cases cited, and since there is at present no way of evaluating the extent of such a hindrance to recombination, the reactions will for convenience of description be considered as taking place directly between iron and carbon monoxide.

The rate of reaction of certain aromatic compounds can be predicted theoretically (cf. Dewar, 1949), but no information appears to be available for any haem compounds. The results presented above now make it possible to appraise these particular systems comparatively, taking the rate of formation of carbon monoxide–haem as a standard. The rates of reaction will depend upon the availability of the valency electrons of the iron atom, which will participate in the bond with carbon monoxide, and this will be affected by the nature of the haem itself as well as by the nature of the conjugated group which is not dissociated by light. (Carbon monoxide is itself intensely reactive owing to the fact that the electronic configuration of the carbon atom is depleted, whereby the molecule acquires an electron-acceptor or cationoid character.)

Since the only difference between protohaem and protopyridine haemochromogen lies in the replacement of a water molecule by a pyridine group, this must be the cause of the marked difference in the velocity constants of the two forms. The pyridine molecule is strongly cationic by comparison with water, i.e. it tends to attract electrons towards itself. It appears therefore that the direct linkage of pyridine to the central iron atom reduces the accessibility of the valency electrons to carbon monoxide (as shown by the reduction in the velocity constant), by the attraction which it exerts upon those electrons. Assuming that there actually is combination with a water molecule after photolysis, it could be considered that a 'tighter' bond was being formed, i.e. that the volume of the electron cloud was being reduced through the effect of the pyridine. The same phenomenon is observed with natural myoglobin (Smith & Gibson, 1959). The two rates are in fact virtually identical, suggesting that the effect is already maximal even upon combination with such a relatively small molecule as pyridine.

A similar argument can be applied to the differences observed within each group, where the structure of the individual haem is being varied. The haems differ only in the constitution of the side chains at positions 2 and 4 of the porphyrin ring, namely, vinyl, ethyl and hydrogen respectively for proto-, meso- and deutero-haem. Such groups may be arranged in a series in which the cationoid character progressively increases. The three groups of immediate interest can be arranged in the series hydrogen < ethyl < vinyl. Hence protohaem, which contains two vinyl groups, might be expected to show the slowest reaction rates owing to the attraction exerted on the valency electrons through the structure of the molecule. Similarly, deuterohaem should show the fastest rates since

hydrogen atoms will exert little if any effect; mesohaem should be slower than deuterohaem. This relationship does apply to the pyridine haemochromogen reactions, which have rates in the ratio of 1:2·5:3·1. (The myoglobin reactions give anomalous results in the mesomyoglobin reaction.) The free haem reactions at first sight appear to be anomalous in that meso- and deutero-haem apparently have the same rate, although this is still much faster than that of protohaem. However, it must be noted that these reactions are extremely fast, being of the same order of magnitude as some of the peroxidase reactions (Chance, 1949), and it is possible that the two fastest rates may require some revision when further improvements in technique become possible. It may be that there is a similar progression with the free haems but that this is obscured by the present difficulties in measuring the very fastest rates.

One obvious drawback in applying such an interpretation to individual haems is the electromeric effect, known to occur in the vinyl group, whereby electrons may be donated to other parts of the molecule by resonance. This would oppose the inductive effect invoked above, and is usually found to be more important. On the other hand, the differences between the observed reaction rates are not great enough to rule out a predominating inductive effect in this instance. It is to be hoped that further work with substituted haems will be undertaken to resolve this point. For the present it appears that variation in the inductive attraction of the side groups represents one characteristic property of the molecule which can account for the observed data; and this suggestion is put forward as a basis for further investigation.

SUMMARY

1. Spectrophotometric observations of haem and carbon monoxide–haem in aqueous solution and in ethylene glycol suggest that carbon monoxide–haem is monomeric in both solvents, whereas haem itself is dimeric in aqueous solution but is depolymerized in ethylene glycol.

2. The dissociation curve of carbon monoxide–haem in aqueous solution was found to be a rectangular hyperbola. Different haem solutions gave 50% saturation at 0·75 and 3·5–4·5 mm.Hg carbon monoxide at 20°. The material also obeyed Beer's law over the range of carbon monoxide concentration 0·075–65 μM.

3. The photochemical absorption spectrum of carbon monoxide–haem indicated that only a part of the material was being dissociated on flash photolysis. A new peak appeared at 420 mμ, corresponding in position to the peak of monomeric haem in glycol. It is suggested that this new peak is caused by the absorption spectrum of the haem nucleus subsequent to the photodecomposition.

4. The dissociation of pyridine haemochromogen shows three stages. Spectrophotometric evidence supports the belief that three compounds of haem and pyridine are formed consecutively, the last of which is the well-known 'pyridine haemochromogen'. It is suggested in explanation of these observations that two of the compounds are dimeric, only 'pyridine haemochromogen' itself being monomeric.

5. The rate of combination of carbon monoxide with three closely related haems was measured by the flash-photolysis technique. The rates of combination with proto-, meso- and deutero-haem respectively were found to be approximately 16, 85 and 85 μM^{-1} sec.$^{-1}$ in 0·02N-sodium hydroxide at room temperature.

6. The rate of combination of carbon monoxide with the pyridine haemochromogens of the same three haems was similarly measured. The rates were found to be respectively 0·42, 1·04 and 1·30 μM^{-1} sec.$^{-1}$ under the same conditions.

7. A theoretical explanation of the variation in these results, and also of those reported in the next paper, is attempted.

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REFERENCES

Chance, B. (1949). *Arch. Biochem.* **22**, 224.
Dewar, M. J. S. (1949). *The Electronic Theory of Organic Chemistry*. Oxford: Clarendon Press.
Drabkin, D. L. (1942). *J. biol. Chem.* **146**, 605.
Drabkin, D. L. & Austin, J. H. (1935). *J. biol. Chem.* **112**, 89.
Fischer, H. & Gibian, H. (1941). *Liebigs Ann.* **548**, 183.
Gibson, Q. H. (1959). *Biochem. J.* **71**, 293.
Hill, R. (1926). *Proc. Roy. Soc.* B, **100**, 419.
Hill, R. (1929). *Proc. Roy. Soc.* B, **105**, 112.
Hogness, T. R., Zscheile, F. P., jun., Sidwell, A. E., jun. & Barron, E. S. G. (1937). *J. biol. Chem.* **118**, 1.
Keilin, J. (1949*a*). *Biochem. J.* **45**, 440.
Keilin, J. (1949*b*). *Biochem. J.* **45**, 448.
Keilin, J. (1950). *Nature, Lond.*, **165**, 151.
Krebs, H. A. (1929). *Biochem. Z.* **204**, 343.
Lemberg, R. & Legge, J. W. (1949). *Hematin Compounds and Bile Pigments*. New York: Interscience Publishers Inc.
Lewis, G. N., Goldschmid, O., Magel, T. T. & Bigeleisen, J. (1943). *J. Amer. chem. Soc.* **65**, 1150.
Lewis, G. N., Magel, T. T. & Lipkin, D. (1942). *J. Amer. chem. Soc.* **64**, 1774.
Maehly, A. C. & Åkeson, A. (1958). *Acta chem. scand.* **12**, 1259.

Rabinowitch, E. & Epstein, L. F. (1941). *J. Amer. chem. Soc.* **63**, 143.
Schalfejeff, M. (1885). *J. Soc. phys.-chim. russe*, p. 30. Quoted in *Ber. dtsch. chem. Ges.* **18** (Referate) 232.
Shack, J. & Clark, W. M. (1947). *J. biol. Chem.* **171**, 143.
Smith, M. H. & Gibson, Q. H. (1959). *Biochem. J.* **73**, 101.
Treibs, A. (1933). *Liebigs Ann.* **506**, 196.
Walter, R. I. (1952). *J. biol. Chem.* **196**, 151.
Wang, J. H., Nakahara, A. & Fleischer, E. B. (1958). *J. Amer. chem. Soc.* **80**, 1109.
Warburg, O. & Negelein, E. (1929). *Biochem. Z.* **204**, 495.
Weber, G. & Teale, F. W. J. (1958). *Trans. Faraday Soc.* **54**, 640.

The Preparation and some Properties of Myoglobin Containing Meso- and Deutero-Haem

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Although experiments in which the protohaem of haemoglobin was replaced by other haems or porphyrins were first carried out by Hill & Holden (1926), the number of papers dealing with similar experiments is comparatively small, and even fewer have been concerned with the quantitative study of reactions of these 'synthetic' haemoproteins. Myoglobin, unlike haemoglobin, is free from effects due to haem–haem interaction and therefore seems particularly suited for work on the relation between the constitution of the haem group and the properties of the haemoprotein, yet little work has been reported. Drabkin (1945) has described the reconstitution of myoglobin with protohaem and has found that the reconstituted material has the same spectral absorption and crystal habit as the native protein. The preparation and properties of reconstituted myoglobin have recently been more fully described by Theorell & Åkeson (1955).

Since this work was completed a preliminary report has appeared from Rossi-Fanelli & Antonini (1957), who have prepared native human myoglobin apo-protein and have coupled it with protohaem and deuterohaem. They report the spectra of various derivatives, and found that there was a threefold increase in oxygen affinity on replacing protohaem with deuterohaem.

The object of the present experiments was to prepare myoglobins containing meso- and deuterohaem, to examine their spectra and to investigate the kinetics of their combination with carbon monoxide. A preliminary account has been given by Gibson & Smith (1957).

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EXPERIMENTAL

Haemins. The haemins used were prepared as described in the preceding paper (Smith, 1959).

Sodium dithionite. A sample of Manox dithionite was obtained from Holdman and Harden, Miles Platting, Manchester, 1.

Buffers. Phosphate buffers, pH 6·5–7·5 (Sörensen, 1909); for pH 5·0–6·3, 0·2 N-citric acid–0·2 N-NaOH (Britton & Welford, 1937); for pH 7·8–9·1, 0·2 M-H_3BO_3 in 0·2 N-KCl with 0·2 N-NaOH (Clark, 1928).

Whale myoglobin. Whale meat from a local fishmonger was prepared both by the methods of Theorell & de Duve (1947) and of Bowen (1948). No crystals were obtained from the samples of whale meat available to us by either of these methods, although considerable yields of myoglobin were obtained in amorphous form.

Horse-heart myoglobin. Horse hearts were obtained fresh from the local abattoir and prepared by the method of Bowen (1948). Four hearts were used at various times, of which three yielded the typical wheatsheaf clusters of crystals at the final stages of the preparation. The fourth gave amorphous material only.

Preparation of native globin. Haematin was removed from the myoglobin molecule by a method devised by Dr F. W. J. Teale (personal communication). A solution of metmyoglobin was dialysed for 24 hr. against running distilled water in a rocking dialyser in 0·5 in. cellophan tubing. To 1 vol. of an approximately mM-solution of the salt-free myoglobin at 0°, 0·5 vol. of 2-butanone containing 10% (v/v) of 0·1 N-HCl was added. The mixture was shaken gently and allowed to stand for 15 min. If a layer of 2-butanone had separated, it was removed. A second portion of acid butan-2-one equal in volume to the first was then added and the mixture again shaken and allowed to stand for 15 min. The process of removal of 2-butanone and its replacement with a fresh portion was continued until no further haematin passed into the upper layer. The lower solution of globin was dialysed against water or 0·02 M-phosphate buffer, pH 7·1. The solution was stable for several days when stored at 0° and pH 7·1.

Reconstitution of myoglobin. A solution of the required strength of haematin (approximately 0·5 mM) in 0·02 N-NaOH was added gradually to a roughly equimolar solution of globin at 0° with stirring. The pH was measured with a glass electrode and a few drops 0·1 N-HCl were added, if necessary, to keep the mixture below pH 6·0 until all the haem had been run in. The mixture was then brought to between pH 7·0 and 8·0 by the addition of 0·1 N-NaOH. A precipitate which appeared on the first addition of the NaOH disappeared again as the addition of alkali continued. To precipitate denatured myoglobin and uncombined globin or haem the solution was next dialysed against half-saturated $(NH_4)_2SO_4$ solution, prepared by mixing equal volumes of saturated $(NH_4)_2SO_4$ and water and neutralizing with a few drops of aq. 7% (w/w) NH_3 solution. The precipitate was removed by centrifuging and the supernatant dialysed against distilled water or 0·02 M-phosphate buffer, pH 7·1, to remove $(NH_4)_2SO_4$. The resulting solutions were used either directly or after dilution for spectroscopic observation and for kinetic experiments.

Spectrophotometry. The concentration of myoglobin solutions was determined with a Unicam SP. 600 spectrophotometer, Drabkin's (1947) millimolar extinction coefficient $\epsilon^{mM}_{540\,m\mu} = 11{\cdot}3$ being used for metmyoglobin cyanide. Drabkin (1942) found that the maximum extinction coefficients for several derivatives of meso- and copro-haem had the same numerical values as those for the corresponding derivatives of protohaem. It seemed reasonable to assume that the extinction coefficients for meso- and deutero-metmyoglobin cyanide had also the numerical value $\epsilon^{mM} = 11{\cdot}3$. The wavelengths used were 530 mμ for mesometmyoglobin cyanide and 534 mμ for deuterometmyoglobin cyanide. Similarly, the value of Hogness, Zscheile, Sidwell & Barron (1937) for the extinction coefficient of haematin $\epsilon^{mM}_{490\,m\mu} = 6{\cdot}55$ was used for all three haematins, readings being taken at 482 mμ for meso- and deutero-haematin. In determining the concentration of globin, $\epsilon^{mM}_{286\,m\mu} = 16{\cdot}2$ was used (Dr F. W. J. Teale, personal communication).

Kinetic experiments. These were carried out with the apparatus and methods described by Gibson (1956).

RESULTS

Spectral findings

The wavelengths of maximum absorption and the millimolar extinction coefficients for natural and reconstituted myoglobins are shown in Table 1. Additional columns have been included which give the results of Theorell (1934), Kiese & Kaeske (1942) and Bowen (1949), who have published figures for protomyoglobin. Except for the results of Kiese & Kaeske, as quoted by Bowen, the agreement between the various authors is satisfactory. Theorell's (1934) values are particularly close except for the γ-band of metmyoglobin. These values have been calculated from the specific extinction coefficients given by Theorell, taking the molecular weight of myoglobin as 17 300. Theorell & Åkeson (1955) have given new values for the molar extinction coefficients of protomyoglobin, all of which are in satisfactory agreement with those given here. They have at the same time recalculated the earlier figures, to express these as molar extinction coefficients. The recalculated figures are now about 10% lower than those given in Table 1, and are at the same time about 10–15% lower than the latest figures.

This divergence appears to be due to the interpolation of two different values for the molecular weight of myoglobin in Theorell's calculations. The 1934 figures appear to have been recalculated with an earlier value (Theorell, 1932) of 16 500 for the molecular weight (the present authors used 17 300), whereas the 1955 values are apparently based on the latest value of 18 800 for the molecular weight. This distinction is not made clear by Theorell & Åkeson, and largely explains the difference between individual values which they have attributed to iron impurities in the 1934 preparation.

The figures given by the present authors in Table 1 represent in every case the means of at least three determinations with different preparations of myglobin. In no case did the range between different preparations exceed 10% of the quantity measured, and was usually considerably less.

Kinetic results

Protomyoglobin. Experiments were first carried out to see if the native and reconstituted materials would yield similar values for the combination velocity constant for carbon monoxide. Both whale-muscle and horse-heart myoglobins have been used, but in view of the failure to obtain crystalline myoglobin from whale muscle, fewer experiments were carried out with it. The results of reconstitution experiments with whale myoglobin are given in Table 2. The reconstituted material combined slightly less rapidly than the native protein. With three preparations of horse-heart myoglobin the results were variable, the reconstituted myoglobin giving a markedly lower velocity constant than the native protein in two cases. In comparing the meso- and deutero-myoglobin with protomyoglobin, therefore, direct comparison of the three reconstituted proteins was preferred to comparison of native protomyoglobin with meso- and deutero-myoglobin. When the splitting and reconstitution procedure was repeated, with separate portions of a single preparation of native myoglobin, each portion of reconstituted myoglobin gave the same velocity constant for combination with carbon monoxide. The differences between preparations recorded in Table 2 are believed to be due to differences in the original myoglobin preparations rather than to variations of the experimental procedures for splitting and reconstitution.

Table 1. *Wavelengths of absorption maxima and millimolar extinction coefficients for myoglobin derivatives*

	Horse-heart protomyoglobin						Meso-myoglobin		Deutero-myoglobin		Whale proto-myoglobin	
	Theorell (1934)*		Kiese & Kaeske† (1942)		Bowen (1949)		Gibson & Smith‡ (1957)					
	mμ	ϵ^{mM}	mμ	ϵ^{mM}	mμ	ϵ^{mM}	mμ	ϵ^{mM}	mμ	ϵ^{mM}	mμ	ϵ^{mM}	mμ	ϵ^{mM}
Metmyoglobin	630	4·0	630	2·7, 7·2	630	3·7	630	3·9	—	—	626	—	630	4·7
	500	8·8	500	8·9, 17·0	500	9·8	500	9·5	492	—	500	10·7	500	9·6
	407	116·0§	—	—	—	—	408	153·0	394	102·0	392	130·0	410	148·0
	280	28·9	—	—	—	—	—	—	—	—	—	—	—	—
Metmyoglobin cyanide	—	—	—	—	540	11·3‖	540	11·3‖	530	11·3‖	534	11·3‖	540	11·3‖
							422	117·1	412	102·8	410	96·2	422	119·0
Reduced myoglobin	555	11·2	555	13·4, 29·8	555	12·9	552	13·0	546	13·0	542	12·1	556	14·9
	435	107·2	—	—	—	—	432	114·0	422	117·5	422	95·5	434	123·8
CO–myoglobin	579	12·6	580	11·6, 17·5	577	12·9	580	12·3	556	12·2	560	12·2	578	15·1
	540	12·3	540	12·2, 19·2	540	14·8	542	15·0	528	15·3	530	15·2	540	17·0
	424	162·6	—	—	—	—	422	167·0	409	111·0	408	176·0	426	187·0
	340	20·8	—	—	—	—	—	—	—	—	—	—	—	—
	275	36·3	—	—	—	—	—	—	—	—	—	—	—	—
Oxymyoglobin	582	12·6	586	17·0	582	15·1	582	13·4	—	—	—	—	582	15·2
	542	12·3	547	17·5	544	14·6	544	13·9	—	—	—	—	545	15·2
	418	95·1	—	—	—	—	420	112·0	—	—	—	—	418	129·5

* Theorell (1934) gives the following specific extinction coefficients $\left(\log \frac{I_0/I}{cd}\right.$, where c = g./l. and d = 1 cm.$\left.\right)$: reading down the column, 0·23, 0·51, 6·7, 1·67, 0·65, 6·2, 0·73, 0·71, 9·4, 1·2, 2·1, 0·73, 0·71 and 5·5. The millimolar extinction coefficients are calculated from these, taking the mol.wt. of myoglobin as 17 300, after Bowen (1948).

† The first column gives values calculated by us as described in the text; the second column is taken from Bowen (1949).

‡ Values of the extinction coefficient in these columns are obtained by first finding the concentration of the solution as the ferric cyanide compound, Drabkin's (1947) value of 11·3 being used for the millimolar extinction coefficient at 540 mμ.

§ George & Irvine (1952) give a value of 163·5 for this coefficient.

‖ Drabkin's (1947) millimolar extinction coefficient of 11·3 at 540 mμ is commonly accepted.

Table 2. *Reactions of native and reconstituted myoglobins with carbon monoxide*

Figures are the velocity constants [M^{-1} sec.$^{-1}$ × 10^{-6}] for the reaction myoglobin + CO → myoglobin–CO, measured by flash photolysis. The columns refer to separate preparations of myoglobin, and the figures in parentheses give the numbers of reconstitution experiments or separate experimental determinations averaged in each case. Protein concentration 20 μM; CO concentration 200 μM. Temperatures were between 18° and 22° in each case. pH 7·1, 0·2M-phosphate buffer.

	Horse			Whale	
Native	0·53 (1)	0·46 (1)	0·43 (1)	0·72 (3)	0·74 (4)
Protomyoglobin	0·52 (3)	0·28 (2)	0·14 (5)	0·54 (1)	0·64 (7)
Mesomyoglobin	—	0·24 (2)	0·17 (6)	—	0·66 (1)
Deuteromyoglobin	1·29 (4)	1·16 (1)	0·69 (2)	—	1·25 (4)

The results for the native protein confirm the species difference suggested by Gibson (1956), the velocity constants for horse-heart myoglobin being appreciably smaller than those for whale myoglobin. Millikan's (1936) finding that the rate constant is independent of pH within the range 7·0–9·0 has been confirmed. It has been found, however, that in more acid solutions there is a moderate increase which reaches 50% at pH 5·0.

Mesomyoglobin. The kinetic results with mesomyoglobin given in Table 2 show that the change from vinyl to ethyl side chains on the porphyrin molecule has apparently had no effect on the rate constant. The reconstituted protomyoglobin combines at the same speed as mesomyoglobin in each of the samples examined.

Deuteromyoglobin. The results of experiments with deuteromyoglobin are also included in Table 2. In this case it has been found that the replacement of the vinyl groups of protohaem by hydrogens to give deuterohaem has led to a marked increase in the rate of reaction with carbon monoxide. The average increase in rate is between 2½- and 3-fold, as compared with the reconstituted protomyoglobin from the same preparation.

DISCUSSION

Up to the present only two series of hybrid haemoproteins, in which the native protein is combined with various haems, appear to have been described. These are the reconstituted haemoglobins (Hill & Holden, 1926; Warburg & Negelein, 1932) and the reconstituted peroxidases (Gjessing & Sumner, 1942; Theorell, Bergstrom & Åkeson, 1942). Although Drabkin (1945) has previously reported the reversible splitting of protomyoglobin, this is believed to be the first time that myoglobins other than those occurring naturally have been prepared. Because of its theoretical simplicity myoglobin is often used as a model on which to base theories of haemoglobin reactions, and it is thus of considerable interest to know that myoglobin, too, will form a series of hybrid compounds.

The spectral data presented for meso- and deutero-myoglobin show a general shift of the bands towards the blue, behaviour which is parallel to that shown by simpler haem compounds (Drabkin, 1942) and with the findings of Hill & Holden (1926) for mesohaemoglobin. The figures given for protomyoglobin agree well with Theorell's findings (1934; Theorell & Åkeson, 1955). Kiese & Kaeske (1942) have published tracings obtained with a recording spectrophotometer for (among other compounds) carbon monoxyhaemoglobin, nitric oxide haemoglobin, methaemoglobin and haemoglobin; carbon monoxymyoglobin, nitric oxide myoglobin, metmyoglobin and myoglobin. Their records were taken in pairs and compared directly the corresponding derivatives of myoglobin and haemoglobin. Bowen (1949) has calculated extinction coefficients from these curves, reproduced in Table 1, which differ widely from those of other authors. These discrepancies appear to be due to errors in the labelling of the ordinates of the spectrophotometric records. If, instead of calculating the extinction coefficients from the ordinates, the extinction coefficients for the haemoglobin derivatives are taken from Lemberg & Legge (1949), then the curves for myoglobin may be evaluated with the ratio of haemoglobin concentration to myoglobin concentration given by Kiese & Kaeske. The results so obtained have been added to Table 1 and are in reasonable agreement with Theorell's, Bowen's and our own figures, making it clear that the discrepancy is of arithmetic rather than of observational origin. The source of Bowen's entry for Kiese & Kaeske's observations on oxymyoglobin is obscure since this compound was not among the derivatives they examined.

Kinetic studies on haematin compounds have been concentrated on materials such as haemoglobin, catalase and peroxidase, and myoglobin has received little attention. The classical paper on myoglobin kinetics is that of Millikan (1936), which until quite recently gave the only published value for the velocity of combination of carbon monoxide with native myoglobin. With a flow method he obtained a value of $(0{\cdot}3 \pm 0{\cdot}1) \times 10^6\,\text{M}^{-1}\,\text{sec.}^{-1}$ at 20°. More recently Gibson (1956) obtained a value of $(0{\cdot}55 \pm 0{\cdot}03) \times 10^6\,\text{M}^{-1}\,\text{sec.}^{-1}$ at 19° for whale myoglobin. In the present studies native whale myoglobin was found to give a velocity constant of $(0{\cdot}69 \pm 0{\cdot}04) \times 10^6\,\text{M}^{-1}\,\text{sec.}^{-1}$ at 18–22°, whereas three preparations from horse heart gave a mean value of $(0{\cdot}47 \pm 0{\cdot}03) \times 10^{-6}\,\text{M}^{-1}\,\text{sec.}^{-1}$ at 20·5°. Gibson pointed out the differences between his result and that of Millikan, and suggested that a species difference might exist. Although the present experiments have confirmed Gibson's previous observations on whale myoglobin it now appears that the species difference is less than had been supposed, the result with horse-heart myoglobin being somewhat higher than that suggested by Millikan. Since Millikan's experiments were carried out with a carefully checked and very reliable flow method, it is likely that his lower values reflect real differences in the preparations, some of which were stored for considerable periods before use.

One of the chief difficulties in the present work has been the occurrence of erratic and unpredictable changes in myoglobin preparations on storage. One sample of crystalline whale myoglobin prepared by Dr S. R. Elsden and Dr F. W. J. Teale in 1955, and examined at intervals by flash-photolysis

and by flow methods, has given the same velocity constant from the time of its preparation up to the time of writing. This sample has been stored as a crystalline paste at $-12°$. On the other hand, a sample of crystalline horse-heart myoglobin also stored at $-12°$ showed a decrease of 50% in the rate of combination with carbon monoxide after 6 months of storage. This material showed no sign whatever of changes in the absorption spectrum which might be associated with denaturation. In general our experience has been that most samples gave lower combination rates after storage, and some preparations have shown a significant decrease after storage for as little as 2 weeks. These effects cannot easily be explained, but may perhaps be due to small differences in the preparative procedure initiating molecular changes which proceed further in the isolated protein even in the cold. It appears that changes in the rate of combination with carbon monoxide provide a much more sensitive means of detecting incipient denaturation in the protein than do spectral changes or other properties of the material.

In many cases the splitting and reconstitution procedures have caused similar changes in the velocity constant for the combination of myoglobin. The extent of these changes seems to have depended more on the initial state of the myoglobin than upon the splitting and reconstitution procedure itself, since in several samples the reconstituted material has had the same or almost the same velocity constant as the original material. Further, where considerable changes have taken place on splitting and reconstitution, these changes have remained constant in size from one batch of the same preparation to another and so are probably due, not to variations in the procedures themselevs, but to degenerative changes which had taken place in the myoglobin preparations used.

Although it was the exception rather than the rule to obtain a reconstituted material with the same kinetic properties as the native protein, the fact that such a result could be obtained at all is evidence that the haem group can be returned to an environment little different from that from which it was taken. This result has some bearing on the 'crevice' theory of St George & Pauling (1951), suggesting that the 'crevice' is more in the nature of a dimple, since the haem molecule could scarcely be expected to penetrate deeply within the body of the myoglobin molecule unless the removal of the haem group is associated with unfolding of the peptide chain, which is reversed on resynthesis of the haemoprotein. The suggestion that the site of the haem group is rather near the surface appears to be in agreement with the latest results of the detailed X-ray studies of Kendrew, Bodo, Dintzis, Parrish & Wyckoff (1958).

Although the difficulties in comparing the native with the reconstituted myoglobins are very considerable, it seems possible to draw definite conclusions from the comparison of the three synthetic myoglobins, as, in spite of variations in the rate of combination of any one myoglobin with carbon monoxide, due presumably to the state of the globin used in its preparation, the comparative picture has remained the same. Proto- and mesomyoglobin have similar rates of combination, whereas deuteromyoglobin reacts considerably faster, the average value for the increase in rate on substituting deuterohaem for protohaem being about 3½-fold. In the preceding paper (Smith, 1959) it is shown that in the carbon monoxide haemochromogens the rates of reaction of the proto-, meso- and deutero-haem compounds with carbon monoxide are in the ratio 1:2·5:3·1. The observed rate must be influenced by the side chains at positions 2 and 4 of the porphyrin ring, since this is the only point of difference between the compounds. The side chains concerned are vinyl, ethyl and hydrogen in proto-, meso- and deuteroporphyrin respectively, and the electron-attracting power of these groups decreases in the same order. Thus the side chains of deuterohaem have the least influence on the valency electrons of the iron atom, whereas the vinyl groups of protohaem may exert a stronger attraction on them through the structure of the haem. The former might therefore be expected to show a faster reaction rate than the latter, since the combining electrons of the iron would be more accessible to the ligand molecule in this case. Although this is observed experimentally in the haem compounds mentioned above, in the myoglobins mesomyoglobin forms an exception to the simple rule suggested. There is no reason why the behaviour of the haemoprotein must reflect that of the simpler compounds. The problem is exceedingly complex and is unlikely to be resolved until further information is available about the nature of the myoglobin molecule and of the haem–globin linkage. From the functional point of view, the results suggest that a fairly specific haem structure is required to maintain the normal combination rates, and it may be tentatively supposed that in the myoglobins it is the removal of one or more of the carbon atoms belonging to the side chains in positions 2 and 4 of the porphyrin nucleus, rather than the exchange of an unsaturated for a saturated grouping, which has the greatest effect on the reaction rate. This suggests that in this case a steric rather than an electromeric effect is the more important. The order of effect found with the different forms of myoglobin is similar to the changes obtained when meso- and deutero-peroxidase were compared with protoperoxidase by Theorell *et al.* (1942), who found

activities of 57 and 63% respectively as compared with that of the native protein.

SUMMARY

1. The preparation and spectra of native, reconstituted proto-, meso- and deutero-myoglobin are described.

2. The rates of combination of these proteins with carbon monoxide have been measured. In most cases the rate for the reconstituted proto-myoglobin was lower than that for the native protein.

3. The relative rates for reconstituted proto-, meso- and deutero-myoglobin were 1:1:3·5.

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REFERENCES

Bowen, W. J. (1948). *J. biol. Chem.* **176**, 747.
Bowen, W. J. (1949). *J. biol. Chem.* **179**, 235.
Britton, H. T. S. & Welford, G. (1937). *J. chem. Soc.* p. 1848.
Clark, W. M. (1928). *The Determination of Hydrogen Ions*, 3rd ed., p. 200. Baltimore: Williams and Williams.
Drabkin, D. L. (1942). *J. biol. Chem.* **146**, 605.
Drabkin, D. L. (1945). *J. biol. Chem.* **158**, 721.
Drabkin, D. L. (1947). *J. biol. Chem.* **171**, 409.
George, P. & Irvine, D. H. (1952). *Biochem. J.* **52**, 511.
Gibson, Q. H. (1956). *J. Physiol.* **134**, 112.
Gibson, Q. H. & Smith, M. H. (1957). *J. Physiol.* **136**, 27*P*.
Gjessing, E. G. & Sumner, J. B. (1942). *Arch. Biochem.* **1**, 1.
Hill, R. & Holden, H. F. (1926). *Biochem. J.* **20**, 1326.
Hogness, T. R., Zscheile, F. F., jun., Sidwell, A. E., jun. & Barron, E. S. G. (1937). *J. biol. Chem.* **118**, 1.
Kendrew, J. C., Bodo, G., Dintzis, H. M., Parrish, R. G. & Wyckoff, H. (1958). *Nature, Lond.*, **181**, 662.
Kiese, M. & Kaeske, H. (1942). *Biochem. Z.* **312**, 121.
Lemberg, R. & Legge, J. W. (1949). *Hematin Compounds and Bile Pigments*. New York: Interscience Publishers Inc.
Millikan, G. A. (1936). *Proc. Roy. Soc.* B, **128**, 366.
Rossi-Fanelli, A. & Antonini, E. (1957). *Arch. Biochem. Biophys.* **72**, 243.
St George, R. C. C. & Pauling, L. (1951). *Science*, **114**, 629.
Smith, M. H. (1959). *Biochem. J.* **73**, 90.
Sörensen, S. P. L. (1909). *Biochem. Z.* **21**, 131.
Theorell, H. (1932). *Biochem. Z.* **252**, 1.
Theorell, H. (1934). *Biochem. Z.* **268**, 55.
Theorell, H. & Åkeson, A. (1955). *Ann. Acad. Sci. fenn.* II*a*, **60**, 303.
Theorell, H., Bergstrom, S. & Åkeson, A. (1942). *Ark. Kemi Min. Geol.* A16, no. 13.
Theorell, H. & de Duve, C. (1947). *Arch. Biochem.* **12**, 113.
Warburg, O. & Negelein, E. (1932). *Biochem. Z.* **244**, 9.

The Lipids of Whole Blood

1. LIPID BIOSYNTHESIS IN HUMAN BLOOD *IN VITRO*

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In recent years the erythrocyte has been shown [Caffrey, Tremblay, Gabrio & Huennekens (1956), and Lowy, Ramot & London (1958)] to possess at least some of the normal metabolic pathways, contrary to the older view which considered the cell simply as an inert carrier of haemoglobin (for a recent review see Prankherd, 1955). Little detailed study, however, has been made of lipid turnover, though Lovelock (1955) suggested on the basis of physical measurements that the structural integrity of the erythrocyte is maintained by its own metabolic activity. Altman, Watman & Salomon (1951) described the incorporation *in vivo* of [*Me*-^{14}C]acetate into the stroma of the rabbit erythrocyte and suggested that metabolically active lipid constituents of the stroma (with a life span of 3–4 days) exchange with chemically identical plasma components. This type of exchange had previously been demonstrated by Muir, Perrone & Popják (1951) to occur with erythrocyte cholesterol. Altman (1953) reported the incorporation *in vitro* of [*carboxy*-^{14}C]acetate into the stroma of the rabbit erythrocyte during the incubation, either of whole blood or of erythrocytes in saline; approximately 1·9% of the total activity was found in the stroma after 6 hr. Activity of a comparable order was also found in the fatty acids of the plasma lipids. Similar incorporations *in vitro* of labelled acetate were demonstrated to occur in human blood by Altman & Swisher (1954).

We have extended this work and have demonstrated the ability of the cellular components of blood to synthesize a wide variety of fatty acids, to incorporate these acids into both triglycerides and phospholipids and to exchange both lipids with

certain of the plasma lipoproteins. Preliminary accounts of this work have already been published (James, Lovelock & Webb, 1957, 1958).

EXPERIMENTAL

Blood was withdrawn from volunteers by venepuncture directly into sterile citric acid–sodium citrate–dextrose (Lovelock, 1955). All incubations were carried out in sterile apparatus in the presence of antibiotics. In a typical experiment 10 ml. of whole blood was added to 1·5 ml. of citric acid–sodium citrate–dextrose with the following additions: [*Me*-^{14}C]acetate (10 μc; 0·73 mg.), penicillin (1000 units), streptomycin (1000 units) and ethylenediaminetetra-acetic acid (50 μg.) and incubated in a centrifuge pot on a roller-tube apparatus at 37° in air.

At the end of the incubation period (normally 5 hr.) the red and white cells were removed by centrifuging and then washed with 3 ml. of 0·9% NaCl soln., the washings being added to the plasma. The cells were resuspended in 0·9% NaCl and added in a thin stream to 20 vol. of stirred ether–ethanol (1:2, v/v). The extraction was allowed to proceed at room temperature for 18 hr. The plasma was divided into two fractions by the addition of dextran sulphate, by the method of Oncley, Walton & Cornwell (1957). The precipitate produced consisted of β-lipoprotein and probably some of the chylomicra or the lower-density lipoproteins. The soluble portion is referred to in this paper as the α-lipoprotein fraction. The precipitate was removed by centrifuging and both plasma fractions were extracted with ether–ethanol overnight.

After filtration, the ether–ethanol lipid solution was evaporated to dryness *in vacuo* and extracted twice with acetone. The insoluble material is referred to as phospholipid and the soluble portion as neutral fat. Phosphorus analyses showed that the soluble fraction contained very little phospholipid.

In one experiment the neutral fat from both α-lipoproteins and erythrocytes was separated into cholesterol esters, triglyceride and free sterol by chromatography in a liquid–solid system, with silica gel (Mallinckrodt) as the adsorbent and with the solvent system described by Hirsch & Ahrens (1958). The neutral fat (89 mg.) was applied to the column (1·1 cm. diam. by 30 cm.; 18 g. of adsorbent) in a small volume of light petroleum (b.p. 40–60°). The cholesterol esters (39 mg.) were eluted with 350 ml. of 1% (v/v) ether–light petroleum and 60 ml. of 4% (v/v) ether–light petroleum, the triglycerides with 350 ml. of 4% (v/v) ether–light petroleum and the free sterols with 10% (v/v) ether–light petroleum. Two further fractions, possibly consisting of diglyceride and small amounts of phospholipid, were eluted with ether and then with acetone. Both fractions contained bound fatty acids, as did the free sterol fraction. The lipid fractions were saponified by heating under reflux with methanolic 10% KOH for 2 hr. At the end of this period the methanol was removed *in vacuo*, water was added and the non-saponifiable material was removed by extraction (twice) with light petroleum. After acidification with 5N-H_2SO_4 the long-chain fatty acids were extracted with ether. The solution of fatty acids in ether was dried with anhydrous Na_2SO_4, evaporated to dryness and esterified by refluxing with dry methanolic 0·8N-HCl for 2 hr. The procedures of application of the sample to the gas chromatogram and counting adopted for the isolated fractions have been described previously (James, Peeters & Lauryssens, 1956). Where complete separation of acids was not achieved, e.g. linoleic acid and oleic acid, only parts of the zones were collected so that contamination of fractions was avoided. The gas chromatogram used for most of the work had Apiezon M grease as the stationary phase so that linoleic acid and linolenic acid were not separated. The counting was carried out with a conventional end-window counter registering 50 000 counts/min. with a standard of 1 μc of ^{14}C. Acids of as yet unknown structure are included in this study (see James & Webb, 1957).

The [*Me*-^{14}C]acetate used in these experiments was checked for the presence of higher fatty acids of microbiological origin by adding 1 μc to a sample of unlabelled plasma lipids. The mixture was subjected to the normal process of extraction, saponification and esterification and the methyl esters of the fatty acids isolated were separated on the gas chromatogram. No activity could be detected in any of the acids.

Incubation in 0·9% sodium chloride. Cells were removed from whole blood by centrifuging, washed twice with 0·9% NaCl and resuspended in this solution with the same additions as described for whole blood. At the end of 5 hr. the red and white cells were removed, washed and extracted with ether–ethanol as before. The 0·9% NaCl supernatant was also extracted. No labelled long-chain fatty acids could be found in this supernatant.

RESULTS

In the early experiments, in which [*Me*-^{14}C]acetate was incubated with sterile whole blood for 5 hr., isolation of the erythrocyte and plasma phospholipids and neutral fat showed appreciable radioactivity in all fractions, as described by Altman (1953). Isolation of the fatty acids from these lipids showed most of this activity to be due to the long-chain fatty acids. In Tables 1 and 2 are shown comparisons of specific activities of the major fatty acid components of these lipids, in experiments with blood from three subjects. Tables 3 and 4 give the fatty acid compositions of the lipids studied. Maximal activity was found in the plasma neutral fat but this could not be due to any synthetic activity of the plasma itself since incubation of labelled acetate with blood after removal of the red and white cells produced very little incorporation into either neutral fat or phospholipid. (This residual incorporation could be due to the platelets remaining.) This synthesis of long-chain acids is therefore carried out by the red and possibly the white cells, which produce both neutral fat and phospholipid, which are then secreted into the plasma.

Reference was made in an earlier publication (James & Lovelock, 1956) to the great variety of fatty acids in the human erythrocyte. All the odd-number straight- and branched-chain acids and unsaturated acids up to a chain length of 20 carbon atoms that exist as minor components were labelled as well as those described in Table 1, but

in this paper attention is directed only towards the major components.

It cannot yet be stated with certainty whether the activity of the mixed peak of linoleic acid and linolenic acid is due to labelling of one or both acids. However, the fact that the activity of arachidonic acid in general follows that of the two-component peak suggests that linoleic acid is labelled.

In two of the experiments reported above, sufficient material was available for separation of the plasma lipoproteins into two main groups by precipitation of the β-lipoprotein with dextran sulphate. The two fractions were extracted and the extract was split into neutral fat and phospholipid. In Table 5 is given the fatty acid composition of the four fractions obtained from two blood samples.

Table 1. *Comparison of specific activities of isolated fatty acids from erythrocyte lipids*

—, Not determined; 0, zero value; m.u., mono-unsaturated.

(Counts/min./mg.)

	Erythrocyte neutral fat		Erythrocyte phospholipids		
Acid	Subject 1	Subject 3	Subject 1	Subject 2	Subject 3
n-C_{10}					
n-C_{12}	—	325	—	—	255
n-C_{14}	64	660	67	174	506
m.u. C_{16}		25	—	—	69
n-C_{16}	176	132	31	52	41
Di- + tri-unsat. C_{18}	0	0	0	5	34
m.u. C_{18}	104	60	81	27	37
n-C_{18}	400	203	87	54	87
Unsat. 'C_{19}A'	—	—	—	13	78
Arachidonic	—	—	—	7	18
Poly-unsat. C_{20}	—	—	43	70	84

Table 2. *Comparison of specific activities of isolated fatty acids from plasma lipids*

—, Not determined, as quantity was too small; 0, zero value; m.u., mono-unsaturated.

(Counts/min./mg.)

	Plasma neutral fat			Plasma phospholipid		
Acid	Subject 1	Subject 2	Subject 3	Subject 1	Subject 2	Subject 3
n-C_{10}	—	—	1300	—	—	1070
n-C_{12}	1620	1365	1030	—	310	760
n-C_{14}	661	416	520	89	185	612
m.u. C_{16}	275	63	102	—	9	129
n-C_{16}	682	590	180	25	54	570
Di- + tri-unsat. C_{18}	36	42	61	7	27	31
m.u. C_{18}	170	68	377	32	22	40
n-C_{18}	284	272	312	43	98	122
Unsat. 'C_{19}A'	—	—	—	—	—	—
Arachidonic	—	—	—	12	20	21
Poly-unsat. C_{20}	—	—	—	31	41	83

Table 3. *Fatty acid composition of erythrocyte lipids in Table* 1

—, Not determined, as quantity was too small; 0, zero value; trace, less than 1%; m.u., mono-unsaturated. Composition is given as percentages. Major components only are listed.

	Erythrocyte neutral fat		Erythrocyte phospholipid		
Acid	Subject 1	Subject 3	Subject 1	Subject 2	Subject 3
n-C_{10}	0	0·6	0	0	0
n-C_{12}	1·1	2·2	0	0·1	0·2
n-C_{14}	17·3	5·9	2·5	1·9	1·2
m.u. C_{16}	3·2	6·0	Trace	1·10	1·6
n-C_{16}	15·2	22·6	25·2	23·7	23·0
Di- + tri-unsat. C_{18}	21·1	11·4	9·1	12·3	8·6
m.u. C_{18}	29·1	28·8	17·0	19·0	25·5
n-C_{18}	5·7	10·7	16·6	19·2	17·7
Unsat. 'C_{19}A'	Trace	Trace	Trace	5·7	2·2
Arachidonic	8·3	7·4	22·3	12·3	10·0
Poly-unsat. C_{20}	Trace	Trace	4·1	2·4	4·1

Table 4. *Fatty acid composition of plasma lipids in Table 2*

—, Not determined, as quantity was too small; 0, zero value; trace, less than 1%; m.u., mono-unsaturated. Composition is given as percentages. Major components only are listed.

	Plasma neutral fat			Plasma phospholipid		
Acid	Subject 1	Subject 2	Subject 3	Subject 1	Subject 2	Subject 3
n-C_{10}	0·2	0	0·2	0	0	0·1
n-C_{12}	0·9	0·7	0·6	Trace	0·3	0·4
n-C_{14}	10·2	10·9	4·3	2·9	2·6	2·0
m.u. C_{16}	2·2	5·4	4·5	1·7	2·4	4·0
n-C_{16}	21·2	22·4	26·0	28·0	23·3	17·0
Di- + tri-unsat. C_{18}	12·5	11·5	6·6	17·2	19·5	18·7
m.u. C_{18}	25·1	30·1	43·8	16·0	19·5	32·3
n-C_{18}	8·3	7·4	10·7	14·4	14·1	8·4
Unsat. 'C_{19}A'	Trace	Trace	—	Trace	0·6	Trace
Arachidonic	8·0	2·4	—	11·6	7·7	9·4
Poly-unsat. C_{20}	4·0	Trace	—	3·4	5·6	5·0

Table 5. *Fatty acid composition of lipids of plasma α- and β-lipoproteins*

—, Not determined, as quantity was too small; 0, zero value; trace, less than 1%; m.u., mono-unsaturated; br., branched (*iso-* or *ante-iso*). Composition is given as percentages. Major components only are listed.

	α-Lipoprotein				β-Lipoprotein		
	Neutral fat		Phospholipid		Neutral fat + phospholipid	Neutral fat	Phospholipid
Acid	Subject 1	Subject 2	Subject 1	Subject 2	Subject 1	Subject 2	Subject 2
m.u. C_{12}	0	0·3	0·3	0	0·6	0	0·5
n-C_{12}	0·4	0·8	0·2	0	0·5	2·2	0·5
m.u. C_{14}	1·4	2·5	0·8	5·1	2·2	6·8	2·8
n-C_{14}	2·5	3·7	0·8	3·6	2·1	3·6	0·9
n-C_{15}	1·1	1·1	1·0	Trace	0·9	Trace	Trace
m.u. C_{16}	4·9	5·2	0·8	7·1	3·2	5·0	2·6
n-C_{16}	18·5	25·7	29·6	37·8	24·0	19·8	31·3
br. C_{17}	2·2	1·8	1·3	Trace	Trace	1·1	4·3
n-C_{17}	Trace	Trace	1·4	Trace	Trace	Trace	Trace
Di- + tri-unsat. C_{18}	16·3	13·4	18·1	14·8	7·9	10·5	10·2
m.u. C_{18}	38·8	35·8	15·1	15·3	16·8	18·4	17·4
n-C_{18}	8·1	7·6	16·4	16·4	23·1	10·7	18·5
Unsat. 'C_{19}A'	1·8	Trace	Trace	—	—	—	Trace
Arachidonic	2·9	Trace	11·5	—	—	—	6·2
Poly-unsat. C_{20}	Trace	Trace	2·5	—	—	—	5·0

Table 6. *Comparison of specific activities of fatty acids isolated from lipids of α- and β-lipoproteins*

—, Not determined, as quantity was too small; 0, zero value; m.u., mono-unsaturated. Results are in counts/min./mg.

	α-Lipoprotein				β-Lipoprotein	
	Neutral fat		Phospholipid		Neutral fat + phospholipid	Phospholipid
Acid	Subject 1	Subject 2	Subject 1	Subject 2	Subject 1	Subject 2
n-C_{12}	—	1800	—	—	—	—
m.u. C_{14}	856	520	378	—	—	96
n-C_{14}	6800	4600	515	—	437	520
m.u. C_{16}	164	172	—	—	—	—
n-C_{16}	1520	1045	30	124	81	87
Di- + tri-unsat. C_{18}	54	123	0	0	—	—
m.u. C_{18}	355	120	18	—	—	388
n-C_{18}	900	1015	61·0	148	—	533
Arachidonic	322	—	20	—	—	88
Poly-unsat. C_{20}	—	—	77·0	—	—	192

Table 7. *Specific activities of the components of the α-lipoprotein neutral fat separated on a silicic acid chromatogram*

Eluting solvent	Fraction no.	Percentage of total extract	Composition	Counts/min./mg. (uncorrected)
Ether–light petroleum (b.p. 40–60°) (1%, v/v)	1	47·4	Cholesterol esters + hydrocarbon	0·5
Ether–light petroleum (b.p. 40–60°) (4%, v/v)	2	17·1	Triglycerides + some free sterol	48
Ether–light petroleum (b.p. 40–60°) (10%, v/v)	3	7·3	Free sterol + unknown lipid	82
Ether	4	3·6	Unknown	52
Acetone	5	8·4	Unknown	19

Table 8. *Fatty acid composition of components of the neutral fat of α-lipoproteins*

0, Zero value; trace, less than 1%; m.u., mono-unsaturated; br., branched (*iso-* or *ante-iso*); h.br., highly branched. Composition is given as percentages.

Acid	Fraction no. (see Table 7) 1	2	3	4	5
n-C_7	2·0	0	0	0	0
n-C_8	0	0	9·6	5·1	Trace
br. C_9	0	0	0	Trace	5·7
n-C_9	0	0	6·0	6·1	10·9
n-C_{10}	0	0·1	4·3	1·6	2·3
h.br. C_{11}	0	0	0	1·8	0
br. C_{11}	0	0	0	0	3·7
h.br. C_{12}	0	0	0	2·0	4·1
m.u. C_{12}	0	0·4	0	0	0
n-C_{12}	Trace	2·2	37·0	9·7	29·3
h.br. C_{13}	0	0	0	2·9	0
h.br. C_{14}	0	0	0	6·4	0
n-C_{14}	1·1	5·6	11·2	8·0	12·2
h.br. C_{15}	0	0	0	10·0	0
br. C_{15}	Trace	0·5	Trace	Trace	Trace
n-C_{15}	Trace	0·92	Trace	Trace	Trace
h.br. C_{16}	0	0	0	9·5	0
m.u. C_{16}	3·6	4·5	0·5	0	0
n-C_{16}	9·4	25·9	7·6	7·7	11·5
h.br. C_{17}	0	0	0	7·3	0
h.br. C_{18}	0	0	0	4·2	0
Di- + tri-unsat. C_{18}	56·5	10·9	5·4	4·0	2·5
m.u. C_{18}	22·0	40·5	11·0	8·4	13·0
n-C_{18}	0	7·7	2·6	2·6	4·8

Table 9. *Specific activities of fatty acids isolated from components of the neutral fat of α-lipoprotein*

Results are in counts/min./mg. —, Not determined, as quantity was too small; h.br., highly branched; m.u., mono-unsaturated.

Acid	Fraction no. (see Table 7) 1	2	3	4	5
n-C_{12}	No activity	—	27	—	4
n-C_{14}		311	208	—	15
n-C_{16}		181	94	21	10
h.br. C_{17}		—	—	13·0	—
Di- + tri-unsat. C_{18}		Low	Low	—	—
m.u. C_{18}		4	7	—	Low
n-C_{18}		62·0	34·0	40·0	—

Table 10. *Specific activities of components of the neutral fat of the erythrocyte*

Fraction no. (see Table 7)	Percentage of total extract	Composition	Counts/min./mg. (uncorrected)
1	25·2	Cholesterol esters + hydrocarbon	14
2	6·4	Triglycerides + some free sterol	549
3	61·3	Free sterol + unknown lipid	7·0
4	<1	Unknown	Labelled
5	3·8	Unknown	106

Table 11. *Specific activities of fatty acids isolated from components of the neutral fat of the erythrocyte*

—, Not determined, as quantity was too small; h.br., highly branched; m.u., mono-unsaturated. Results are given in counts/min./mg.

	Fraction no. (see Table 7)			
Acid	1	2	3	5
h.br. C_{12}	No activity	46	—	—
n-C_{12}		113	—	—
n-C_{14}		595	—	32
m.u. C_{16}		110	—	—
n-C_{16}		352	11	6
Di-unsat. C_{18}		51	—	—
m.u. C_{18}		151	—	—
n-C_{18}		700	20	—
Arachidonic		169	—	—
Poly-unsat. C_{20}				

Table 12. *Comparative composition of neutral fat fractions from erythrocytes (see Table 10)*

—, Not determined, as quantity was too small; 0, zero value; trace, less than 1%; br., branched; h.br., highly branched; m.u., mono-unsaturated.

	Fraction no. (see Table 7)			
	1	2	3	5
Acid	(%)	(%)	(%)	(%)
br. C_6	0	0	High, not estimated	0
n-C_6	0	0		0
br. C_7	0	0		0
n-C_7	0	0		0
br. C_8	0	0		0
n-C_8	0	0	1·8	0
br. C_9	6·0	0	0	0
n-C_9	0·2	3·2	21·9	12·5
br. or unsat. C_{10}	0·6	0	Trace	1·1
n-C_{10}	1·2	0·3	3·3	1·5
h.br. C_{11}	0·8	0	Trace	Trace
br. C_{11}	0·4	0	1·9	0·7
n-C_{11}	1·2	0	0	4·4
h.br. C_{12}	0·6	1·6	6·4	Trace
n-C_{12}	1·7	2·7	14·2	2·5
br. C_{13}	Trace	1·0	0	6·5
n-C_{14}	2·3	6·1	20·2	4·6
h.br. C_{15}	0	1·8	3·9	5·7
br. C_{15}	Trace	0·3	Trace	Trace
n-C_{15}	Trace	0·3	Trace	Trace
m.u. C_{16}	2·2	3·2	0·9	1·8
n-C_{16}	20·9	21·6	10·0	18·6
br. + unsat. C_{17}	Trace	0·8	2·7	4·6
n-C_{17}	Trace	0·4	Trace	Trace
Di-unsat. C_{18}	25·4	11·1	3·3	3·4
m.u. C_{18}	21·0	31·7	5·9	21·4
Isomers of oleic	2·2	3·7	Trace	Trace
n-C_{18}	8·8	8·3	4·0	10·5
Arachidonic	4·8	1·2	—	—
Poly-unsat. C_{20}	—	0·8	—	—

Marked differences in composition of the neutral fat and phospholipids of the two fractions can be seen; in particular the neutral fat of the α-lipoprotein contains less palmitic acid and stearic acid, but more oleic acid than the phospholipid.

Table 6 shows the specific activities of the isolated fatty acids. Very high activity is found only in the neutral fat of the α-lipoprotein, the descending order of activity being: α-lipoprotein neutral fat, β-lipoprotein phospholipid, α-lipoprotein phospholipid. The β-lipoprotein neutral fat showed very low activity and is not included as such in Table 6.

The fatty acids of the neutral fat of the α-lipoprotein complex arise from both cholesterol esters and from triglycerides. Insufficient material remained from the earlier experiments for further fractionation of the plasma lipids so a large-scale experiment was carried out with 100 ml. of blood. The fractionation was performed as before with the exception that the α-lipoprotein neutral fat was separated into five fractions by chromatography on silica gel. In Table 7 are summarized the properties of these fractions. The cholesterol esters show barely any activity and the triglycerides are highly labelled, as are the other three fractions of unknown constitution. Table 8 gives the fatty acid composition of these fractions. As expected, the cholesterol esters contain only 10% of saturated acids and are particularly rich in oleic acid and linoleic acid.

In Table 9 are given the specific activities of the acids isolated from all five fractions. No activity could be demonstrated in the acids from the cholesterol esters, but high activity was found in the acids from the triglycerides.

Not all the radioactivity of mixtures of fatty acid esters from these experiments was recoverable in acids in the range C_8–C_{20}. The residual activity was found in material running more slowly through the columns and consisting presumably of C_{20} and C_{22} unsaturated acids, since these are known to be present in plasma lipids.

A similar separation was carried out on the neutral fat isolated from the erythrocytes of the large-scale experiment. In Table 10 are given the specific activities of the five fractions obtained from the silicic acid chromatogram. Again the highest activity is found in the triglyceride fraction; all other fractions did, however, show some degree of labelling. The specific activities of the fatty acids isolated from the fractions are shown in Table 11; the acids from the triglyceride fraction have higher activities than those found in the similar fraction from the α-lipoproteins. However, the fatty acid composition of this fraction is similar to that of the triglyceride fraction from the α-lipoprotein, whereas the cholesterol esters from these two lipids are quite different in composition (Table 12).

Tables 13 and 14 show the specific activities of the non-saponifiable materials isolated from the fractions of the α-lipoprotein and erythrocyte neutral fat. Some degree of labelling exists in all except fraction I, which consists of cholesterol esters. The free cholesterol forming the contaminant of fraction 2 is, however, labelled, supporting the view that the cells in human blood are unable to synthesize cholesterol esters but can synthesize cholesterol.

Composition of the neutral-fat fractions

The neutral fat from both erythrocytes and the α-lipoprotein was resolved into five fractions by means of a liquid–solid chromatogram.

Fraction 1. This fraction was eluted under conditions that define it as a mixture of cholesterol esters and hydrocarbons. The fatty acids and non-saponifiable material from both sources were unlabelled. This fraction accounted for 47% of the neutral fat in the α-lipoprotein and 25% in the erythrocyte.

The fatty acid composition of this cholesterol ester fraction from the α-lipoprotein was that expected (Table 8), being largely linoleic acid and oleic acid with only 9·4% of palmitic acid and no stearic acid whatsoever. The similar fraction from the erythrocyte possessed a quite different fatty acid composition (Table 12), with appreciable amounts of the saturated acids, suggesting little or no exchange of this material between erythrocytes and α-lipoprotein.

Fraction 2. This fraction (triglyceride) from both sources was appreciably labelled and contained a

Table 13. *Non-saponifiable material of fractions of α-lipoprotein neutral fat*

Fraction no. (see Table 7)	Percentage of non-saponifiable material	Specific activity (counts/min./mg.) (uncorrected)
1	64 (2·5 mg.)	0
2	< 5	Labelled
3	65 (3·9 mg.)	7
4	26 (0·8 mg.)	2
5	35 (2·4 mg.)	2

Table 14. *Specific activity of non-saponifiable material from fractions of erythrocyte neutral fat*

Fraction no. (see Table 7)	Percentage of total non-saponifiable material	Specific activity (counts/min./mg.) (uncorrected)
1	3 (0·5 mg.)	0
2	< 5	Labelled
3	67 (27·5 mg.)	3
4	?(< 0·1 mg.)	Labelled
5	< 10 (< 0·1 mg.)	0

small amount (less than 5%) of labelled non-saponifiable material (probably cholesterol) (Tables 13 and 14). The larger part of the activity, however, was due to the fatty acids, the composition being very similar in both cases (Tables 8 and 12); this suggests the concept of a rapid incorporation of the cell triglyceride into the α-lipoprotein. The fact that the individual fatty acids had higher specific activities in the erythrocyte than in the α-lipoprotein (Tables 9 and 11) agrees with the concept of a non-equilibrium state in the exchange process.

Fraction 3. This fraction contains a high proportion of labelled unsaponifiable material (about 65% in both α-lipoprotein and the erythrocyte). This fraction was the major component of the erythrocyte neutral fat (61%) but a minor constituent of the α-lipoprotein neutral fat (7·3%). In both cases the fatty acids were of unusual composition, very large amounts of the short-chain acids being present. Presumably most of this fraction is made up of uncombined sterol, and the saponifiable part would appear to be diglyceride, on the basis of its behaviour on the silicic acid chromatogram. If this is so then this diglyceride cannot be derived from either the cell triglyceride or the cellular phospholipid because of their different fatty acid compositions. The acids were labelled from both sources though their specific activities in the erythrocyte fraction were much lower than those in the α-lipoprotein fraction, unlike fraction 2. Here again is presumably an example of a non-equilibrium exchange.

Fraction 4. This was a minor component in both and contained some labelled non-saponifiable material; the fraction was so small in the erythrocyte that fatty acid determinations could not be carried out. The fraction from α-lipoprotein contained 26% of labelled non-saponifiable material and large amounts of the short-chain acids.

Fraction 5. This also was a minor constituent in both and the fatty acids were labelled, though much less than in other fractions. Traces of activity were found in the non-saponifiable material from the α-lipoprotein fraction.

The chemical nature of fractions 4 and 5 is unknown, but it is clear that part at least of these minor constituents of the α-lipoprotein and the erythrocyte are derived from the same source.

DISCUSSION

These experiments have demonstrated the ability of the cells in blood to take up acetate from the plasma and to synthesize a wide variety of saturated and unsaturated acids. In different experiments, between 2 and 4% of the acetate has been converted into long-chain fatty acids (cf. Altman, 1953), an appreciable amount when account is taken of the total weight of red and white cells in the body. [Acetate has been reported as a constituent of plasma by Fonnesu (1951).] The recovery of labelled acids from both neutral fat and phospholipids of the plasma shows that the cells also synthesize these lipids and secrete them into the plasma, where they are incorporated into the lipoproteins. Maximum activity is found in the triglycerides of the α-lipoprotein complex; next follow the phospholipids of the β-lipoprotein and the phospholipids of the α-lipoproteins; the β-lipoprotein neutral fat shows very little incorporation.

Incubation of red and white cells with labelled acetate in 0·9% sodium chloride instead of plasma showed that synthesis of fatty acids occurred as before, but little activity could be demonstrated in the 0·9% sodium chloride except for that due to acetate. The exchange of lipids with the fluid medium does not therefore occur rapidly unless the lipoproteins are present. The differential exchange with the α- and β-lipoproteins would suggest that in one case the neutral fat occupies the outer layer of the lipoprotein and in the other a less accessible inner space, the phospholipid here being more accessible.

Nature of the acids synthesized

The ability of the erythrocyte to synthesize fatty acids is not surprising, since Kaplan & Lipmann demonstrated in 1948 that human erythrocytes contain appreciable amounts of acetyl-coenzyme A. The amount reported (3–4 units/g. of wet tissue) is only about 3% of that found in rabbit liver. However, the total mass of erythrocytes is of the same order as that of the liver and their large surface area would allow ready exchange of synthesized lipids, not only with plasma lipoproteins but also with surfaces of blood vessels and capillaries. Although the results presented in this paper do not allow calculation of rates of synthesis, they do suggest that the red and the white cells, if they operate *in vivo* as they do *in vitro*, would contribute appreciable amounts of lipid to certain of the plasma lipoproteins. It is not possible at this stage to define the relative biosynthetic role of the reticulocytes, the erythrocytes and the various white cells. Studies are in progress to elucidate these points.

The high relative activity of the stearic acid appearing in the α-lipoprotein triglycerides suggests that the blood cells are one of the major sites of synthesis of this acid. In the unsaturated acids, the highest degree of labelling occurs in oleic acid and the acid described as 'poly-unsaturated C_{20}' (having a retention volume relative to methyl stearate of 1·64). The incorporation of acetate into

this acid may be several times greater than that into arachidonic acid, suggesting that it may have some metabolic importance. The structure of this acid has been partially defined; on reduction, arachidic acid is formed and oxidative degradation has defined one of the double bonds at the 8:9 position and another at either the 14:15 or the 15:16 position. A study of the rate of movement of the intact acid on two types of gas–liquid chromatogram confirms the chain length as 20 carbon atoms and suggests that it possesses three double bonds.

The acid described as '$C_{19}A$' has not been chemically defined in any way beyond demonstrating that it is unsaturated. It has a retention volume relative to methyl stearate of 1·27 under the chromatographic condition defined earlier.

The definite but relatively low radioactivity of the mixed peak of linoleic acid and linolenic acid is of interest since it is generally considered that these acids are 'essential' dietary constituents and are not synthesized in animal tissues. The presence of radioactivity in the molecule does not necessarily imply complete synthesis, since some exchange process is possible; e.g.

$$\text{linoleate or linolenate} \rightleftharpoons \text{fragment} + \text{acetate} \rightleftharpoons \text{acetate pool},$$

as well as a purely synthetic pathway such as

$$\text{palmitoleate} + \text{acetate} \rightleftharpoons \text{linoleate or linolenate}.$$

The nature of the process of incorporation of acetate can only be defined by degradation of the molecule to determine the position of the radioactive atoms. So far the small amounts of material obtained have prevented a complete degradation, but after oxidation labelled azelaic acid was successfully isolated.

Lipid exchange with the plasma lipoproteins

The specific activities of the fatty acids of the phospholipids of the erythrocyte and of both plasma-lipoprotein fractions are closely similar; this would suggest that the process of exchange between the erythrocyte and the plasma is more rapid than that of synthesis in the cell itself; this notion receives further support from the quantitative measurements of phospholipid exchange to be reported in a later paper. By contrast the specific activities of the various fatty acids of the triglycerides of the α-lipoprotein fraction are different from those observed with the cell triglycerides. This would suggest either a rate of synthesis by the red or white cells much more rapid than that of exchange with the plasma, or that the process of exchange with the plasma is irreversible. Quantitative measurements of the exchange process to be reported in a later paper indicate that the former suggestion is the more probable. The evidence so far accumulated is shown schematically in Fig. 1.

The evidence reported in this paper indicates that the cells in human blood have a remarkable capacity for lipid synthesis. In the wide variety of fatty acids isolated from the various lipid fractions all or almost all included isotopic carbon; this would suggest that human blood is a particularly convenient and suitable tissue for study of the various processes concerned in the metabolism of fatty acids.

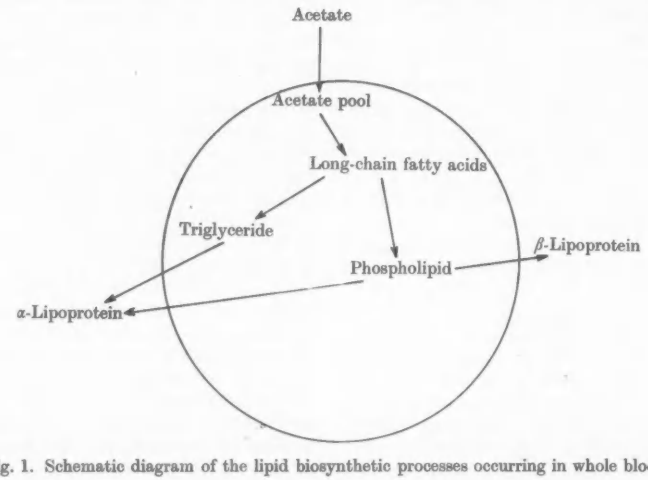

Fig. 1. Schematic diagram of the lipid biosynthetic processes occurring in whole blood.

SUMMARY

1. The cellular components of human blood are shown to be a convenient system for studies of fatty acid and lipid synthesis and exchange.

2. After incubation of whole blood with [*Me*-^{14}C]acetate all the common saturated and unsaturated acids are found to be labelled, including the mono- and poly-unsaturated acids of a variety of chain lengths.

3. The 'essential' fatty acids arachidonic acid, and linoleic acid or linolenic acid or both, are also labelled.

4. The long-chain fatty acids are incorporated into triglycerides and some neutral lipids of undefined structure, as well as into phospholipids, but not into cholesterol esters.

5. The triglycerides and other neutral lipids are rapidly secreted into plasma, where they are incorporated into the plasma α-lipoproteins but not into β-lipoprotein.

6. The phospholipids are more readily incorporated into the plasma β-lipoproteins.

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REFERENCES

Altman, K. I. (1953). *Arch. Biochem. Biophys.* **42**, 478.
Altman, K. I. & Swisher, S. N. (1954). *Nature, Lond.*, **174**, 459.
Altman, K. I., Watman, R. N. & Salomon, K. (1951). *Arch. Biochem. Biophys.* **33**, 168.
Caffrey, R. W., Tremblay, R., Gabrio, B. W. & Huennekens, F. M. (1956). *J. biol. Chem.* **223**, 1.
Fonnesu, A. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 1021.
Hirsch, J. & Ahrens, E. H. (1958). *J. biol. Chem.* **233**, 311.
James, A. T. & Lovelock, J. E. (1956). *The Blood Lipids and the Clearing Factor*. Brussels: Koninklijke Vlaamse Academie voor Wetenschappen.
James, A. T., Lovelock, J. E. & Webb, J. (1957). *Biochem. J.* **66**, 60.
James, A. T., Lovelock, J. E. & Webb, J. (1958). In *Essential Fatty Acids*, p. 72. Ed. by Sinclair, H. M. London: Butterworths Scientific Publications.
James, A. T., Peeters, G. & Lauryssens, M. (1956). *Biochem. J.* **64**, 726.
James, A. T. & Webb, J. (1957). *Biochem. J.* **66**, 515.
Kaplan, N. O. & Lipmann, F. (1948). *J. biol. Chem.* **174**, 37.
Lovelock, J. E. (1955). *Biochem. J.* **60**, 692.
Lowy, B. A., Ramot, B. & London, I. M. (1958). *Nature, Lond.*, **181**, 324.
Muir, H. M., Perrone, J. C. & Popják, G. (1951). *Biochem. J.* **48**, 1.
Oncley, J. L., Walton, K. W. & Cornwell, D. G. (1957). *J. Amer. chem. Soc.* **79**, 4666.
Prankherd, T. A. J. (1955). *Brit. J. Haematol.* **1**, 131.

The Localization of ^{131}I in Biosynthetically Labelled [^{131}I]Thyroxine

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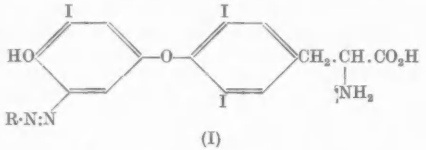
Work in this Laboratory on the biosynthesis and peripheral metabolism of thyroid hormones necessitated a method for the degradation of thyroxine whereby the proportions of radioiodine present on each of the two aromatic rings could be determined. The diazo-coupling reaction described in this paper was the best of several chemical reactions investigated with a view to displacing iodine specifically from the ring distal to the alanine side chain. Thyroxine, a phenolic coupling component, has no unsubstituted position *ortho* or *para* to the hydroxyl group. On coupling therefore iodine must be displaced from organic combination or the diphenyl-ether linkage is destroyed. The reaction has been studied by other workers, but never in conjunction with isotopic-tracer techniques. Komant (1950) coupled thyroxine with diazotized 2:4-dichloroaniline. Barac & Morren (1953) studied the reaction with sulphanilic acid and *p*-arsanilic acid. They isolated the *p*-arsanilic acid azo-compound in 60% yield and claimed that it was an azo-substituted tri-iodothyronine. It was then apparent that a large proportion of the diazo-coupling occurs by the elimination and replacement of the *ortho* substituent.

At best therefore in a reaction between equimolecular quantities of thyroxine and diazonium salt, where coupling is confined to the *ortho* position, a product of structure (I) is to be expected, where R is a substituted aryl nucleus. If the structural formula is correct, it follows that when [3:5-^{131}I$_2$]-thyroxine is subjected to diazo-coupling, no radioiodine is displaced from organic combination. If the ^{131}I were present on both rings in the same amounts, 25% would be converted into inorganic iodide. On theoretical grounds therefore it is

likely that a linear relationship exists between the percentage of ^{131}I eliminated from the organic molecule, and the percentage of it which was situated in the 3′:5′ positions. This paper shows



(I)

that the distribution of ^{131}I within the molecule of labelled thyroxine may be determined by subjecting the compound to diazo-coupling and measuring the percentage of its radioiodine content which is eliminated from organic combination.

METHODS

Preparation of thyroxine samples. [3′:5′-$^{131}I_2$]Thyroxine was prepared by the method of Gross & Leblond (1950). [3:5-$^{131}I_2$]Thyroxine was prepared by a micromodification of the method of Michel, Roche & Tata (1952). All the [^{131}I]thyroxine samples were purified before use by descending chromatography on Whatman no. 3 MM paper in the solvent butan-1-ol–pentan-1-ol–aq. 2N-NH_3 soln. (1:1:2, by vol.). The thyroxine band was located by radioautography and eluted into ethanol–aq. 2N-NH_3 soln. (1:1, v/v).

Choice of amine. It was clearly desirable that the coupling reaction should occur as extensively as possible, and yet be very selective in its point of attack on the thyroxine molecule. The aim was a quantitative conversion of thyroxine into a homogeneous azotri-iodothyronine product. The work of Komant (1950) indicated that this might be almost obtainable with a 20% excess of 2:4-dichloroaniline. The yield was greatly superior to those claimed for sulphanilic acid and *p*-arsanilic acid (Barac & Morren, 1953).

In some preliminary trials, [3:5-$^{131}I_2$]thyroxine was coupled with 2:4-dichloroaniline and with sulphanilic acid, by the methods described by the above authors. The products were chromatographed on Whatman no. 3 MM paper in the solvent systems butan-1-ol–2N-acetic acid (1:1) and butan-1-ol–dioxan–aq. 2N-NH_3 soln. (4:1:5, by vol.). The positions of the peaks of radioactivity were determined by use of an automatic scanning device. For the coupled product derived from 2:4-dichloroaniline, the greater part of the radioactivity ran as a single peak close to the solvent front in both solvents. Similar chromatographic trials with the sulphanilic acid derivative separated it into multiple components.

2:4-Dichloroaniline was therefore employed in most of the present work, since its coupled product with thyroxine is both obtainable in higher yield and appears to be more nearly homogeneous than that of sulphanilic acid. The term 'diazonium salt' is hereafter used without qualification to denote 2:4-dichlorobenzenediazonium chloride.

Experimental procedure. Stable sodium L-thyroxine (20 mg.) was added to a solution of [^{131}I]thyroxine (0·5 μc) in ethanol–aq. 2N-NH_3 soln. (1:1, v/v), and the sample evaporated to dryness under vacuum. The residue was redissolved in 10 ml. of 0·5N-NaOH, reprecipitated with 0·7 ml. of conc. HCl and the mixture was centrifuged. The supernatant was discarded. The thyroxine precipitate was dissolved in 2 ml. of water and 0·6 ml. of 2N-NaOH and the solution cooled in ice. The diazonium solution was prepared by adding, dropwise and with stirring, 1·7 ml. of 5% (w/v) $NaNO_2$ to a solution of 200 mg. of 2:4-dichloroaniline in 14 ml. of 2N-HCl, all at less than 5°; 2·77 ml. of water was then added. A portion (0·5 ml.) of the diazonium solution was added to the cooled thyroxine solution, drop by drop, with vigorous stirring. The pH was next varied by alternately adding 0·5 ml. volumes of 2N-HCl and of 2N-NaOH. Three complete oscillations of pH were carried out in this way, but the final addition of alkali was doubled in quantity. The sample was then stored at 0° for 16 hr., and the azo-compound was precipitated by addition of 2 ml. of 2N-HCl and centrifuged.

The extreme insolubility of the azo-compound in acid was thus exploited to separate it from inorganic iodide.

Separation. After centrifuging for 15–20 min. at 900 *g* the supernatant fluid was removed and its volume adjusted to 10 ml. The precipitate was then dissolved in 0·2N-NaOH–aq. 40% (v/v) ethanol–aq. 5·4% Teepol 530 (2:1:1, v/v) and the volume of this solution was also adjusted to 10 ml.

Assay of ^{131}I. This was carried out in a Geiger–Müller liquid counting tube (20th Century Electronics, type M6) having a sensitivity of 12 400 counts/min./μc. Five-minute counts were made on both supernatant and precipitate. Although the supernatants and precipitates were dissolved in different solvents, this did not necessitate a correction factor. There was no detectable difference between the absorbance of ^{131}I β-radiation by the two solutions.

RESULTS

The reaction was first carried out on a series of thyroxine samples prepared by mixing together the two radioactively labelled varieties in different, known proportions. The results are shown in Fig. 1. The marked difference between the percentage precipitations obtained with the two kinds of labelled thyroxine confirms the view that iodine is more easily released from the 3′:5′ positions than from the 3:5 positions. However, the slope drawn from the experimental results is not nearly as steep as the theoretical graph.

Thus at this stage the method was effective but inefficient in distinguishing between [3:5-$^{131}I_2$]- and [3′:5′-$^{131}I_2$]-thyroxine. If the standard curve (Fig. 1) ranges from 95% precipitation for [3:5-$^{131}I_2$]-thyroxine to 75% for 3′:5′-labelled thyroxine, an experimental result of 85% should indicate that the thyroxine sample is equally labelled on both rings. Since the measurements are subject to an experimental error of about 2%, however, the correct result for the percentage of ^{131}I in the 3′:5′ positions could lie anywhere between 40 and 60%.

It was accordingly necessary to study the mechanism and conditions of the reaction with a view to improving the slope of the standard curve. The unexpectedly high percentage precipitation

figure for [3′:5′-$^{131}I_2$]thyroxine was found to be due partly to a reaction between the released inorganic iodide and the diazonium salt, in which the radioactivity was reincorporated into the precipitable fraction. When this reaction was studied by coupling stable thyroxine with diazonium salt in the presence of inorganic radioiodide, it was found that 36 % of the radioactivity was precipitated along with the reaction products. This effect could be diminished either by diluting the radioiodide with carrier NaI or by carrying out the reaction in the presence of sodium thiosulphate (Table 1).

When [^{131}I]thyroxine was coupled under these conditions the reaction was found to be more efficient in distinguishing between [3:5-$^{131}I_2$]- and [3′:5′-$^{131}I_2$]-thyroxine (Table 2), but the precipitation figure for [3′:5′-$^{131}I_2$]thyroxine, 65–70 %, still differs seriously from the theoretical figure of 50 %. Side reactions apparently consume much of the diazonium salt and render it unavailable for coupling. It was therefore necessary to employ a larger excess of the diazonium salt to compensate for this loss. Table 3 shows that, provided sufficient thiosulphate is added, more iodine can be displaced in this way from organic combination, thus improving the accuracy of the method.

Use of m-*nitroaniline.* Although 2:4-dichloroaniline couples well at pH values which render thyroxine readily soluble, it cannot be used profitably in quantities greater than 2 moles/mole of thyroxine, because the reaction is terminated prematurely by the precipitation of the coupled products. It was found possible to remedy this defect by replacing 2:4-dichloroaniline with an amine whose azo-compound was less hydrophobic. *m*-Nitroaniline, which proved to be the most

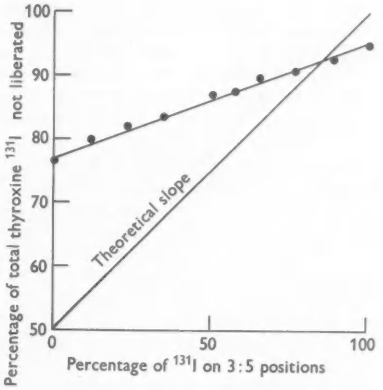


Fig. 1. Precipitation results obtained by coupling 2:4-dichlorobenzenediazonium chloride with the two kinds of [^{131}I]thyroxine, mixed in known proportions. Ordinate values show the percentage of total thyroxine ^{131}I not liberated on diazo-coupling.

Table 1. *Coupling of thyroxine with* 2:4-*dichlorobenzenediazonium chloride: effect of sodium iodide or thiosulphate on the binding of* $^{131}I^-$ *into the precipitable fraction of the reaction products*

Additions made to thyroxine solutions before coupling	Percentage of ^{131}I in ppt.
^{131}I only	36·3
^{131}I + NaI (6·5 mg.)	25·2
^{131}I + NaI (26·0 mg.)	12·1
^{131}I + $Na_2S_2O_3,5H_2O$ (1·2 mg.)	19·2
^{131}I + $Na_2S_2O_3,5H_2O$ (5·4 mg.)	5·5
^{131}I + $Na_2S_2O_3,5H_2O$ (10·8 mg.)	4·6

Table 2. *Coupling of* [^{131}I]*thyroxine with* 2:4-*dichlorobenzenediazonium chloride: effect of sodium iodide or thiosulphate on the release of thyroxine* ^{131}I *from the precipitable fraction of the reaction products*

Additions to thyroxine solutions before coupling	Precipitation (%)		
	[3:5-$^{131}I_2$]Thyroxine	[3′:5′-$^{131}I_2$]Thyroxine	Difference
None	95·0	76·1	18·9
NaI (26·0 mg.)	91·9	67·2	24·7
$Na_2S_2O_3,5H_2O$ (5·4 mg.)	95·6	69·6	26·0

Table 3. *Coupling of* [3′:5′-$^{131}I_2$]*thyroxine with* 2:4-*dichlorobenzenediazonium chloride: effect of excess quantities of diazonium salt on the release of thyroxine* ^{131}I *from the precipitable fraction of the reaction products*

Diazonium salt (moles/mole of thyroxine)	Precipitation (%)		
	No addition	With 2·7 mg. of $Na_2S_2O_3,5H_2O$	With 10·8 mg. of $Na_2S_2O_3,5H_2O$
1·20	76·3	69·6	74·7
1·56	—	65·9	67·5
1·68	76·2	—	—
1·92	—	63·6	63·6
2·20	85·7	70·0	60·8

suitable, could be employed in 260% excess, and the results were the most favourable yet obtained. [3:5-$^{131}I_2$]Thyroxine gave 96% precipitation and [3′:5′-$^{131}I_2$]thyroxine 50%. Since the accuracy of the method had been thus improved by 130% the use of *m*-nitroaniline was adopted as a routine procedure. Complete experimental detail for this modification of the reaction procedure is therefore given below.

Recommended experimental procedure. [^{131}I]-Thyroxine (20 mg.), purified by reprecipitation as described above, is dissolved in 5·2 ml. of 0·463N-NaOH; 0·1 ml. of 0·218M-$Na_2S_2O_3$ is then added and the mixture cooled in ice to less than 5°. The diazonium solution is made by dissolving 215 mg. of *m*-nitroaniline HCl in 10·5 ml. of 2N-HCl, cooling below 5° and mixing in 1·7 ml. of 5% (w/v) $NaNO_2$ dropwise; 1·66 ml. of water is added. Coupling is carried out by addition of 1 ml. of the diazonium solution to the thyroxine solution, drop by drop, with vigorous stirring. The following additions are made to cause pH variations which result in a permanent deepening of the colour of the solution: 0·4 ml. of 2N-HCl, 0·3 ml. of 2N-NaOH, 0·3 ml. of 2N-HCl, 0·3 ml. of 2N-NaOH, 0·3 ml. of 2N-HCl, 0·6 ml. of 2N-NaOH. The mixture is stored overnight (16 hr.) at 0° and the reaction products are finally precipitated with 1 ml. of 2N-HCl. The supernatant fluid is then separated from the precipitate by centrifuging, and this is followed by assay of ^{131}I. The percentage of the ^{131}I of the original thyroxine sample in the 3′:5′ position can then be read off from the standard slope. If percentage precipitation is $73·0 \pm 2·0$ then the percentage of ^{131}I in the 3′:5′ positions is $50·0 \pm 4·5$.

DISCUSSION

The coupling reaction between thyroxine and diazonium salts was described by Komant (1950) as 'atypical', because a pre-existing substituent had to be eliminated from the molecule before coupling could occur. Thyroxine is a fairly complex molecule to employ as a coupling component, and this complexity multiplies the number of theoretically possible ways in which a diazo-compound could react with it. The side chain bears an α-amino group which is available for triazen formation. Furthermore, phenol ethers are known to behave in an exceptional way on coupling; the ether linkage is broken but the azo group does not take up the position which this linkage formerly occupied (Saunders, 1949). The existence of these possibilities, together with simple coupling in the *ortho* or *para* position, indicates that the possible structures for the reaction products in the so-called 'azotri-iodothyronine' precipitate are many and diverse. Although chromatography of the reaction products obtained from 2:4-dichloroaniline failed to separate them into different components, Professor C. Rimington (personal communication) has found that these reaction products can be separated into two different fractions, one of which is readily soluble, and the other only sparingly soluble, in sodium hydroxide solution.

In view of the multiplicity of possible side reactions it is not surprising that when thyroxine and diazonium salt are employed in equimolecular proportions, the displacement of iodine from the 3′:5′ positions is incomplete. Side reactions are apparently active in consuming the diazonium salt well before all the thyroxine has become involved in the reaction. Chromatography of the reaction products and subsequent staining of the chromatograms with ceric sulphate (Bowden, Maclagan & Wilkinson, 1955) showed that even after 1·44 moles of the diazonium salt had been added per mole of thyroxine, some unchanged thyroxine was still readily detectable.

Some interest attaches to the cause of the displacement of iodine from the 3:5 positions. It seems unlikely on theoretical grounds that diazo-coupling can occur in those positions while the diphenylether link is intact. If it were ruptured, however, coupling might well occur readily with the fragment, as it does with 3:5-di-iodotyrosine.

The solubility properties of thyroxine limit the range of amines with which it can be satisfactorily coupled. In order to dissolve the thyroxine, very alkaline conditions are required; this inhibits the coupling reaction by converting the diazohydroxide into an inactive *iso*diazoate. The investigator is therefore restricted to the use of amines whose diazohydroxides are stable at unusually high pH values.

Application of the method. In applying the diazo-coupling method to thyroxine from biological sources, it was necessary to take more stringent precautions for the purification of the samples. These were chromatographed in two or three different solvents before being finally eluted, mixed with carrier and reprecipitated. Not only was it necessary thus to separate the thyroxine from other radioactive metabolites, but much unwanted non-radioactive material was always present which had to be removed lest it should contain phenols or amines capable of coupling with the diazonium salt and thereby consuming it.

Provided that careful chromatographic purification was carried out, reproducible results were always obtainable.

SUMMARY

1. The coupling reaction between thyroxine and diazonium salts has been investigated with thyroxine labelled in either ring.

2. The results indicate that coupling occurs readily in the 3′:5′ positions, with the elimination

of iodine. Coupling in the 3:5 positions occurs only slightly or not at all.

3. This reaction has been utilized as the basis of a method for the localization of ^{131}I in the thyroxine molecule.

4. The method is applicable to biosynthesized [^{131}I]thyroxine.

I am indebted to the Rockefeller Trust of the Medical School and to the Medical Research Council for facilities for carrying out this work.

I wish to thank Miss Diana Walmsley for technical assistance.

REFERENCES

Barac, G. & Morren, H. (1953). *Bull. Soc. Chim. biol., Paris*, **35**, 299.

Bowden, C. H., Maclagan, N. F. & Wilkinson, J. H. (1955). *Biochem. J.* **59**, 93.

Gross, J. & Leblond, C. P. (1950). *J. biol. Chem.* **184**, 489.

Komant, W. (1950). *Hoppe-Seyl. Z.* **286**, 229.

Michel, R., Roche, J. & Tata, J. (1952). *Bull. Soc. Chim. biol., Paris*, **34**, 466.

Saunders, K. H. (1949). *The Aromatic Diazo-compounds*, p. 218. London: Edward Arnold and Co.

The Hydrolysis of Rabbit γ-Globulin and Antibodies with Crystalline Papain

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The molecular size of rabbit γ-globulin is such that any direct attempt to relate structure to biological activity is not feasible at present. An alternative approach is to degrade antibody molecules in such a way that activity will persist in smaller fragments and so to reduce the structural problems involved. In an earlier investigation of this type (Porter, 1950*b*) it was found that, if rabbit γ-globulin containing antiovalbumin was digested with crude papain, a fragment with a molecular weight of about 40 000 could be produced which retained the ability to combine specifically with ovalbumin though it would no longer form a precipitate. It seemed probable that this fragment contained an antibody-combining site.

In that work, the amounts of crude enzyme used relative to γ-globulin were large, making subsequent investigation of the products of digestion rather difficult. As crystalline papain can now be prepared easily (Kimmel & Smith, 1954) and fractionation techniques have improved, these earlier experiments have been repeated. It has been found that γ-globulin is split by papain into three large pieces with very little release of amino acids or small peptides. If the γ-globulin contains antibody against any of the several antigens investigated, then two of these pieces retain combining though not precipitating power. The third piece, which may be readily crystallized, has no antibody activity but it has most of the antigenic sites of the original molecule. The isolation and properties of these three fractions will be described.

A preliminary account of this work has been given (Porter, 1958*a*).

EXPERIMENTAL

Materials

Antisera. Rabbit antiovalbumin, antibovine serum albumin and antihuman serum albumin were prepared by intravenous injection of the alum-precipitated protein (Porter, 1955).

Rabbit antipneumococci polysaccharide type 3 was prepared by injection of a suspension of the formalin-killed bacteria in 0·9% NaCl solution.

Goat antirabbit γ-globulin was prepared by intravenous injection of alum precipitate and subcutaneous injection of γ-globulin with adjuvant (Freund & McDermott, 1942).

Rat antiserum was prepared according to the following schedule. The rats were given two intramuscular injections of protein and adjuvant into different thighs, with 1 week's interval between injection. Five weeks later they were given three injections, with 3-day intervals between injections, of alum-precipitated protein into the tail vein. They were bled by heart puncture 6 days after the last injection.

Rabbit γ-globulin. This was prepared either by chromatography, with diethylaminoethylcellulose (Sober, Gutter, Wyckoff & Peterson, 1956), or by Na_2SO_4 precipitation according to the method of Kekwick (1940).

Crystalline papain. This was prepared from crude enzyme powder purchased from Hopkin and Williams Ltd., London. The enzyme was crystallized once as the free enzyme and twice as the inactive Hg dimer (Kimmel & Smith, 1954). It was freeze-dried and stored as the dimer.

Methods

Quantitative estimation of precipitating antibody. This was carried out according to Kabat & Mayer (1948).

Estimations of inhibitory power. These were made by quantitative antibody assay in the presence of the inhibitor

or by measure of the delay in precipitation caused by the inhibitor with the antibody and antigen mixed in optimum proportions.

Chromatography. Diethylaminoethylcellulose and carboxymethylcellulose were prepared according to Peterson & Sober (1956). Column size was 2·4 cm. (diam.) by 30–35 cm. (ht.); volume of mixing chamber was 1200 ml. Buffers used on carboxymethylcellulose columns were 0·01 M-sodium acetate, pH 5·5, with gradient to 0·9 M-sodium acetate, pH 5·5. In the refractionation of fraction I on diethylaminoethylcellulose, the following system was used: 0·01 M-sodium phosphate, pH 6·4, with gradient to 0·2 M-sodium phosphate, pH 6·2. All buffers were saturated with toluene. The pH was measured with an EIL Direct Reading pH meter (Electronic Instruments Ltd.).

Enzyme digestion. γ-Globulin (150 mg.) and Hg papain (1·5 mg.) were dissolved in 10 ml. of buffer (0·1 M-sodium phosphate, pH 7·0, 0·01 M-cysteine, 2 mM-ethylenediaminetetra-acetate). This solution was incubated at 37° for 16 hr. in the presence of toluene. It was then dialysed against water with vigorous stirring and several changes of the outer liquid over 48 hr. This procedure, which removed the cysteine and ethylenediaminetetra-acetate, and facilitated oxidation, inactivated the enzyme. *N*-Ethyl maleimide was also used to inactivate the enzyme but as there appeared to be no advantage this was not continued. The non-diffusible digestion products were either freeze-dried or fractionated directly by chromatography after dialysis against acetate buffer, pH 5·5.

Protein determinations. Protein concentrations were determined by reading the absorption at 280 and 260 mμ in a 1 cm. cell in a Unicam SP. 500 spectrophotometer.

Radioactivity measurements. Injection of hydrolysed algal [^{14}C]protein and counting of the γ-globulin fraction were carried out as described previously (Askonas, Humphrey & Porter, 1956).

Analytical methods. Amino acid analysis was by the method of Moore & Stein (1951) as modified by McDermott & Pace (1957).

N-Terminal amino acids were estimated by the fluorodinitrobenzene technique (Porter, 1957*a*). Hexose as 'glucose' was estimated by the anthrone method of Mokrasch (1954). Hexosamine as 'glucosamine' was estimated, after separation from amino acids on a cation-exchange resin (Boas, 1953), by a modification of the method of Elson & Morgan (1933).

RESULTS

When a digestion mixture, prepared as described above, was dialysed against water at 2° a precipitate formed which appeared to be crystalline. If, however, the dialysis was against 0·04 N-acetic acid there was no precipitation and the recovery of the non-diffusible digestion products could be estimated either by measuring the absorption at 280 mμ or by freeze-drying and weighing the dry powder. This has been done in a number of experiments, and by either method the recovery of γ-globulin protein taken has fallen in the range 85–95%. In view of the probable handling losses in dialysis and freeze-drying, it is considered that the higher figure is the most accurate. When such a digest was examined in the ultracentrifuge in 0·1 M-phosphate, pH 6·7, some crystals again formed on dialysis but the supernatant showed only one peak ($S_{20,w}$ 3·5). As γ-globulin has $S_{20,w}$ 6·5, it was clear that the protein had been split into large fragments of similar size with very little production of diffusible peptides. Attempts to fractionate this mixture by zone electrophoresis were not successful, but resolution could be achieved by chromatography on carboxymethylcellulose. Acetate buffer, pH 5·5, was chosen because under these conditions most of the carboxyl groups of the ion-exchanger are dissociated; if the digest was brought nearer to neutrality crystals formed, indicating a low solubility of at least one component. To help to keep all the material in solution chromatography was carried out at room temperature (20–23°). With a gradient of increasing salt concentration at this pH, three components could be resolved which have been named fractions I, II and III in order of elution from the column (Fig. 1).

If the gradient on the column was reduced, fractions II and III were more spread and III 'tailed' badly, but there was no suggestion of any further resolution. Fraction I appeared very close to the solvent front and this was re-run on a diethylaminoethylcellulose column at pH 6·4. No fractionation was obtained but again with a slow gradient there was considerable tailing. By these limited criteria the three fractions appeared to be single components and to be the only significant products of the digestion of γ-globulin by papain.

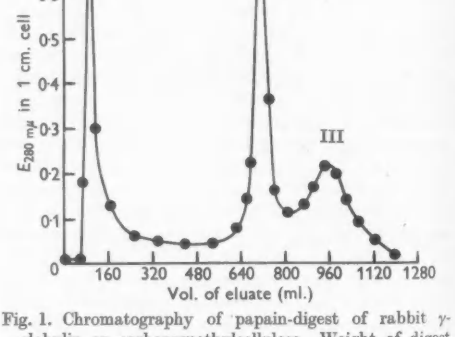

Fig. 1. Chromatography of papain-digest of rabbit γ-globulin on carboxymethylcellulose. Weight of digest 150 mg. Column 30 cm. × 2·4 cm. diam. Volume of mixing chamber 1200 ml. Gradient from 0·1 M-sodium acetate, pH 5·5, to 0·9 M-sodium acetate, pH 5·5, commencing at 200 ml. eluate volume.

Results with shorter times of hydrolysis under the same conditions suggest that the splitting is complete in very much less than 16 hr., the period which has always been used. It follows that these three fractions are exceptionally resistant to further digestion by papain.

Yields of the three fractions were measured by summing the absorption at 280 mμ in each peak. The ratios of yield varied somewhat from experiment to experiment but averaged (I:II:III) 1:0·8:0·9, and total recovery from the column was 85–90%. When re-run, fractions I and III were recovered in about 95% yield and fraction II in about 85% yield. The absorptions of peaks I, II and III at 280 mμ at a concentration of 1 mg./ml. in water or 0·02N-acetic acid were 1·4, 1·4 and 1·0 respectively. If the relative yields are corrected for the lower recovery of fraction II, and the lower specific absorption at 280 mμ of fraction III, then the corrected relative yields (I:II:III) are 1:0·9:1·25 by wt. In some experiments the fractions were concentrated by pressure-dialysis in the cold against water or 0·02N-acetic acid; insoluble material was discarded and the solution freeze-dried and weighed. The yields of I and III were similar to those above but II was somewhat lower. Considerable error can occur because of denaturation in dilute solution, and variable losses on chromatography, but it is considered that the average yields calculated from chromatography approximate to the yields of the fractions produced in the digestion.

Fraction III is readily identifiable as the material with a low solubility near neutrality. It may be crystallized and recrystallized by dialysing a solution in 0·02N-acetic acid against sodium phosphate buffer, pH 6·0–7·0, at 2°. The crystals are diamond-shaped plates, often of considerable size but thin and easily broken (Fig. 2).

Molecular weights. The three fractions were studied in the ultracentrifuge (see Addendum) and the results for normal γ-globulin are summarized in Table 1. The sum of the molecular weights of the three fragments is very close to the molecular weight of the original γ-globulin. This is in agreement with the high recovery of non-diffusible digestion products. The relative sizes of the three fragments were (I:II:III) 1:1·05:1·6, which is in approximate though not exact agreement with the calculated relative yields of the fragments.

Amino acid analysis. Amino acid analysis was carried out by Mr E. E. McDermott and Dr J. Pace at the Cereals Research Association, St Albans. The results are given in Table 2 for fractions prepared from γ-globulin from non-immune serum. The most striking feature of the analysis is the great similarity of the amino acid content of fractions I and II. With the exception of cystine,

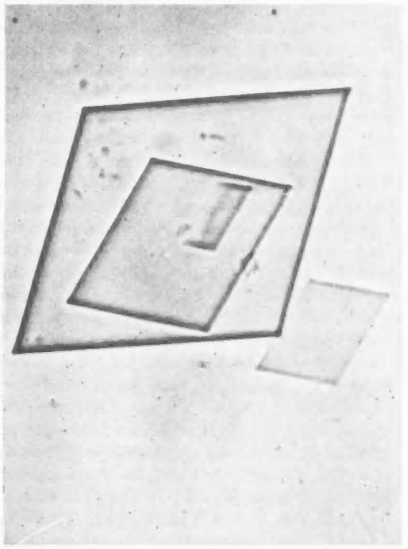

Fig. 2. Crystals of fraction III. Magnification × 500.

Table 1. *Molecular weights of fragments and γ-globulins from non-immune serum*

For details see Addendum.

γ-Globulin	188 000
I	50 000
II	53 000
III	80 000

Table 2. *Amino acid analysis of fragments from non-immune serum*

	N (percentage of total N)		
Amino acid	I	II	III
Aspartic acid	5·66	6·01	6·18
Threonine	10·42	11·56	5·61
Serine	8·77	9·57	7·34
Glutamic acid	5·99	6·18	7·83
Proline	5·08	5·08	6·68
Glycine	7·57	6·93	3·18
Alanine	5·65	6·12	2·82
Valine	7·38	7·58	5·96
Methionine	0·64	0·75	1·48
Isoleucine	2·20	2·26	3·43
Leucine	5·17	5·39	5·19
Tyrosine	3·44	3·58	2·33
Phenylalanine	2·53	2·65	2·76
Histidine	1·59	1·87	5·23
Lysine	6·03	6·84	9·95
Arginine	5·94	6·22	13·93
Tryptophan		Not estimated	
½ Cystine	2·80	2·22	1·18

differences rarely exceed 10 % and, as the results quoted are taken from a single analysis, this is close to the experimental error. In contrast, fraction III shows considerable differences, most marked in the contents of threonine, proline, glycine, alanine, methionine, isoleucine, tyrosine, cystine and the three basic amino acids, the differences in several cases being more than 100 %.

These results have been recalculated in terms of amino acid residues per mol. of fraction and per mol. of γ-globulin. It has been assumed that the ash-free, moisture-free fractions have the same nitrogen content of 16 % as found for γ-globulin by Smith, McFadden, Stockell & Buettner-Janesch (1955). In view of the difference in basic amino acid content the figure may be rather high for fractions I and II and low for fraction III. The results of this calculation are given in Table 3. The amino acid residues accounted for by the three fractions range from 83 to 116 % of the figures for whole γ-globulin. These discrepancies are probably explicable by errors in the different estimations, the assumption of 16 % of nitrogen in each fraction and by the peptide material lost in the dialysis. The overall recovery of amino acid residues in this calculation is 94 %.

Carbohydrate estimations were made by Dr H. R. Perkins of this Institute, and the results with whole γ-globulin and the fractions are given in Table 4. About two-thirds of the carbohydrate of the original molecule is found with fraction III, one third with fraction I and a small amount, which may not be significant, is with fraction II. It seems likely therefore that the carbohydrate moiety of γ-globulin is in the two pieces, the larger with that part of the molecule which corresponds to fraction III and the smaller with the part corresponding to fraction I. If the total recovery of carbohydrate in the three fractions is compared with that present in the original, the yields are approximately 110 and 120 % for hexose and hexosamine respectively.

N-Terminal assay has been carried out on fractions I and II, and in both alanine was found to be the main terminal amino acid (about 0·9 mol./50 000), together with smaller amounts of aspartic acid (0·2 mol./50 000) and trace amounts of serine and threonine (together about 0·1 mol./50 000). This is a very similar result to that obtained for whole rabbit γ-globulin (Porter, 1950*a*; McFadden & Smith, 1955), except that the *N*-terminal amino acid content is more than three times as great as in the whole globulin. It is also in approximate agreement with the results of the digestion of rabbit antiovalbumin with papain powder, when an immunologically active fraction containing one *N*-terminal alanine per 40 000 was reported (Porter, 1950*a*). The significance of finding *N*-terminal alanine in both I and II is not certain, as there are about 110 alanine residues per molecule of γ-globulin. An attempt to determine the *N*-terminal sequence was unsatisfactory owing to shortage of material, but it appeared that the sequence of I was alanylaspartyl and of II alanyl-leucyl. If this is correct, it suggests that II may derive from the *N*-terminal part of the molecule, as the sequence there is alanyl-leucylvalylaspartylglutamyl (Porter, 1950*a*; McFadden & Smith, 1955).

Immunological properties. The three fractions were prepared from digests of γ-globulin which had been obtained from rabbit antisera against ovalbumin, bovine serum albumin, human serum albumin and antipneumococci polysaccharide type 3. None would precipitate with the corresponding antigen but fractions I and II prepared from γ-globulin containing antiprotein antibodies inhibited the precipitation of the antigen by the homologous antiserum. This effect is specific; for

Table 3. *Recovery of amino acid residues*

Amino acid	Total no. of residues in I+II+III*	Total no. of residues in whole γ-globulin†	Recovery (%)
Aspartic acid	126	137	93
Threonine	180	207	87
Serine	175	196	89
Glutamic acid	144	150	95
Proline	121	138	87
Glycine	115	140	83
Alanine	95	111	86
½ Cystine	40	44	91
Valine	144	158	91
Methionine	22	19	116
Isoleucine	58	63	92
Leucine	110	111	100
Tyrosine	63	68	92
Phenylalanine	56	62	90
Lysine	84	83	100
Histidine	23	19	120
Arginine	66	54	120
Tryptophan		Not estimated	
Total	1582	1680	94

* Calculated from molecular weights and amino acid analysis, assuming a nitrogen content of 16 % for each fraction.

† Calculated from Smith, McFadden, Stockell & Buettner-Janesch (1955), assuming a molecular weight of 188 000.

Table 4. *Carbohydrate content of γ-globulin and fractions*

	Hexosamine (%, as glucosamine)	Hexose (%)	Total carbohydrate (%)
γ-Globulin	0·6	0·5	1·1
I	0·5	0·6	1·1
II	0·1	0·15	0·25
III	1·3	0·9	2·2

example, I and II from antihuman serum albumin γ-globulin had no effect when added to bovine serum albumin and its antiserum; fraction III, on the contrary, had no effect whatever its source or whatever the test system. Testing fraction III is difficult owing to its low solubility near neutrality, where antibody–antigen precipitation is carried out, but it is soluble to about 0·3 mg./ml. at 37° at pH 7·2 and it could be shown that it had less than one-thirtieth of the activity of the corresponding fractions I and II.

Quantitative estimates of the inhibitory power of I and II are shown in Fig. 3. In order to assess this on a molar basis it has been assumed that the fractions would have the same proportion of active molecules as the proportion of antibody in the γ-globulin taken, and the weight of each fraction has been corrected accordingly when plotting the graph. On this basis it can be seen that approximately equal weights of inhibitor are required for complete inhibition of precipitation, i.e. a molar ratio of inhibitor to antibody of 3·5:1.

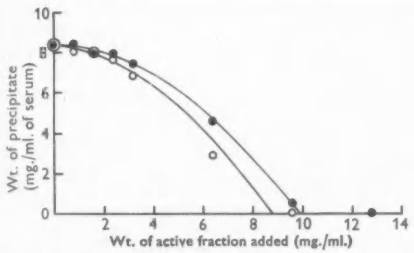

Fig. 3. Inhibition of precipitation of human serum albumin–antihuman serum albumin. Increasing weights of fractions I and II from antihuman serum albumin digest were added to antibody–antigen in equivalence. Weights of fractions were corrected for the presence of inactive material (see text). ●, Fraction I; ○, fraction II.

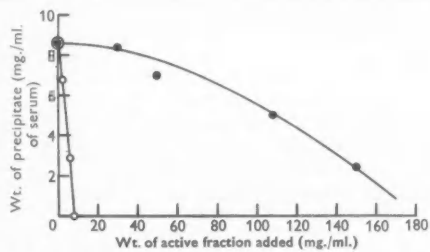

Fig. 4. Inhibition of precipitation of human serum albumin–antihuman serum albumin and pneumococci polysaccharide type 3 antipolysaccharide. ●, Fraction II from antipolysaccharide γ-globulin; ○, fraction II from antiserum albumin γ-globulin.

In contrast with these results none of the fractions from antipolysaccharide γ-globulin appeared to have any power to inhibit the combination of polysaccharide with its antiserum. However, when much higher ratios of inhibitor to antibody were used, inhibition of precipitation occurred with fraction I or II. The difference between the antiprotein and antipolysaccharide fractions appears to be quantitative rather than qualitative, as originally stated (Porter, 1958*a*). The results with fraction II from antipneumococci and antihuman serum albumin γ-globulin are shown in Fig. 4. The inhibitory power of the former is about 4% of that of the latter, on a molar basis. Fraction III again appeared to be completely inactive in qualitative tests, but in this case the solubility limitation is greater and it could only be concluded that III has certainly less than half the activity of I or II.

Antigenic activity. The power of the fractions to precipitate with goat antirabbit γ-globulin serum was now tested and I and II showed neither precipitation nor inhibitory activity. Fraction III precipitated 70% of the antibody precipitated by γ-globulin.

Rat antiserum was prepared against rabbit γ-globulin and fractions I, II and III. Groups of three rats were used for each antigen and, after the immunization course described above, the serum of each group was pooled, and gave antibody contents of 4·2, 7·8, 8·1 and 4·0 mg. of antibody/ml. for γ-globulin, I, II and III respectively. All the fractions were at least as effective antigens as the original molecule, and I and II were the most effective, but as only three animals were used per group the difference may not be significant.

When rat anti-γ-globulin was tested with the fractions, rather different results from those with

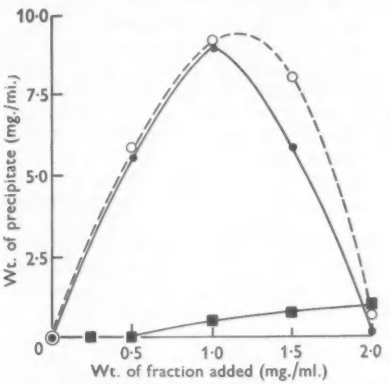

Fig. 5. Precipitation curves of I (●), II (○) and III (■) with rat anti-II serum.

goat antiserum were obtained. Fraction III precipitated 50 % of the antibody, and I and II 15 % each. The antigenic specificities of the fractions were compared by measuring the precipitates formed by I, II and III with rat anti-II serum. The precipitation curves are shown in Fig. 5. It can be seen that the curves for I and II are almost identical, but III gives almost no precipitate. This again emphasizes the great similarity of I and II and their sharp distinction from III.

Synthesis of the fraction in vivo. In view of the distinctive characters of the three fractions the possibility was considered that they might be synthesized separately and joined into the whole molecule in a separate step. If this were to happen the fractions would probably not all be synthesized from the same pool of amino acids at the same time, and hence if radioactive amino acids were injected into a rabbit the incorporation of radioactivity after a short time interval would vary from fraction to fraction. Hydrolysed algal [^{14}C]protein was used as the source of labelled amino acids; 240 μc was injected intravenously, and the rabbit was bled after 1 and 4 hr. The plasma γ-globulin is not labelled until 30 min. after injection (Askonas *et al.* 1956). The γ-globulin was prepared and hydrolysed with papain, and the fractions were isolated, precipitated with trichloroacetic acid, and counted, at infinite thickness on a 1 cm.^{2} disk. The specific activities are given in Table 5 and it can be seen that there is no significant difference between the different fractions. There is therefore no evidence to suggest that these fractions or any large parts of them are synthesized independently.

DISCUSSION

The results show that papain-digestion of rabbit γ-globulin causes limited and highly selective splitting to give three large fragments and very few small peptides. As papain shows rather a wide specificity in the digestion of the β chain of insulin (Sanger, Thompson & Kitai, 1955), it is apparent that the structure of the native molecule must be such that many potentially hydrolysable bonds are protected by steric and other factors. Ultracentrifuge studies of the splitting of γ-globulins from different species, by a variety of enzymes (Petermann & Pappenheimer, 1941; Petermann, 1942, 1946), have all shown that a small number of fragments are the main products in each case. It is possible that in γ-globulins only small parts of the peptide chain are accessible to proteolytic enzymes, so that even though different enzymes break different bonds in these vulnerable sections the principal large digestion products are similar.

Our results with the papain-digestion of rabbit γ-globulin suggest that the single polypeptide chain (Porter, 1950*a*; McFadden & Smith, 1955) has been split into three distinct sections, which together comprise some 90 % of the original molecule. However, fractions I and II are so similar that the question arises whether they could be derived very largely from the same section of the chain. They are almost indistinguishable in *N*-terminal amino acid, amino acid analysis, molecular weight, antigenic specificity and, if derived from antibody, in their antigen-binding capacity. They differ in chromatographic behaviour and carbohydrate and cystine content.

There are at least three possible ways of splitting a single chain to give results such as this, and they are illustrated in Fig. 6: A shows the obvious split into three distinct sections, B the production of two fractions from the same section of chain and C the result if γ-globulin consists of two types of molecule, such as euglobulin and pseudoglobulin, one of which gives rise to I + III and the other to II + III. If either B or C were correct then the yield of III should exceed the sum of the yield of I and II. In fact the yield of each of the three fractions is very similar, as would be expected in A. In B and C the overall recovery, in view of the molecular weights found, would be 130 000/188 000, i.e. 70 %, considerably lower than the experimental figure of about 90 %, which again is

Table 5. *Specific activities of fractions from γ-globulin after injection of algal* [^{14}C]*amino acids*

Standard error was less than 5 %.

Time after injection (hr.)	Counts/min. (on 1 cm.^{2} disk at infinite thickness) I	II	III
1	22	25	25
4	80	84	83

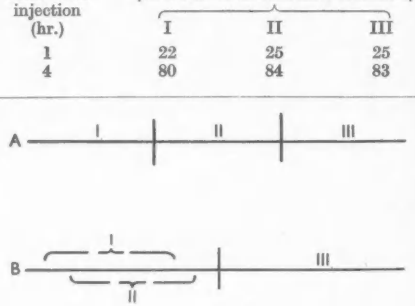

Fig. 6. Different ways of splitting rabbit γ-globulin to give three fractions of approximately equal size. For explanation see Discussion.

that which would be expected if A were correct. Similarly, with the individual amino acid residues, the recoveries would be only 70%, with the possibility of big variations in individual cases as much more material would be lost. In fact the recovery of residues is 90–95%, and the variation 83–120% in the recoveries of individual amino acids is close to the range expected from the errors in the different measurements and the assumptions made in the calculation.

Further, in B it would be expected that if I and II were digested further by papain, either interconversion would occur or both would be reduced to a slightly smaller common product. In fact both appear to be stable to a further 16 hr. digestion with papain at 37°, being unchanged in chromatographic behaviour and other properties. The simplest explanation of our results therefore seems to be that γ-globulin has been split into three distinct fractions, as shown in A. It is probable that the inhibitor described in the earlier work (Porter, 1950*b*) was a mixture of fractions I and II, and that fraction III was prevented from crystallizing by the impurities introduced with the crude enzyme preparation.

It follows that two large sections of γ-globulin (each about 30% of the whole) are extremely similar in chemical, physical and biological properties. The finding of such close agreement between the amino acid analysis of these two fragments and also an almost identical antigenic specificity suggests that there may be almost a repeat of the amino acid sequence and configuration. This is in contrast with the properties of fraction III, which differs in every respect, so that there appears to be a large repeating unit (I or II) joined to a larger section (III) of entirely different character. This unusual make-up of the γ-globulin molecule is presumably related to its antibody activity. The similarity of the pieces with mol.wt., 50 000, where the antibody-combining sites may be very much smaller (Kabat, 1956), raises the question whether large sections are required to maintain the structural integrity of a small combining site or whether antibody-combining sites may occur anywhere in these pieces.

The big quantitative difference between the inhibitory power of fractions I and II, when derived from antiprotein or antipolysaccharide antibodies, may reflect important differences between the two types of combining site, but perhaps more probably arises from differences in the speed and mechanism of precipitation between the two systems.

The significance of the part of the γ-globulin molecule represented by fraction III in antibody–antigen reactions is not known. The ease of crystallization could be taken as evidence of greater identity of structure among individual molecules in III than in the whole molecule, which appears to be complex by all available physical and biological data (see Porter, 1958*b*). Preliminary electrophoretic examination, however, suggests that it is as complex as the original molecule by this criterion. Most of the antigenic sites of γ-globulin appear to be on III, but as these sites can only be defined in relation to a given antiserum (Porter, 1957*b*), variable results might be expected, and there is in fact a difference in this respect between the goat and rat anti-γ-globulin serum. Fraction III is remarkably stable. After precipitation by 5% trichloroacetic acid at room temperature, washing with ethanol and ether and drying for 1 hr. at 110°, it will redissolve in 0·05 N-acetic acid and it retains its power specifically to precipitate goat antirabbit γ-globulin.

The finding that γ-globulin appears to be built of three sections, one of exceptional stability and the other two containing the antibody-combining sites, is reminiscent of Pauling's (1940) theory of antibody formation, in which it is suggested that antibody molecules consist of a rigid centrepiece and two flexible ends capable of taking up configurations complementary to the antigen and hence forming antibody-combining sites on these flexible parts. Pauling further suggested that the flexibility might be due to a high content of proline residues. There is no evidence on the relative positions of fractions I, II and III in the whole molecule, except that II may be from the *N*-terminal end. Nor is there any evidence on the essential feature of Pauling's theory, that the amino acid sequence of all antibodies is identical and that the different antibody-combining sites are formed only by refolding of the same polypeptide chain. The proline content of I and II is less than that of III, whereas the cystine content, often an important feature in determining the stability of a protein molecule, is lower in III than in I and II.

The equal rate of incorporation of radioactive amino acids into the different fractions is in agreement with the view that the whole molecule is synthesized simultaneously from the same pool of amino acids.

SUMMARY

1. Rabbit γ-globulin, when digested by crystalline papain, gives three fragments which together form 90% or more of the original molecule.

2. If the γ-globulin contains antibodies, two fragments (I and II), of molecular weight 50 000–55 000, retain the power to combine with the antigen. The third fragment (III), molecular weight about 80 000, crystallizes easily and has much of the antigenic specificity of the original molecule.

3. I and II are extremely similar in chemical and biological properties, and III differs very widely in all respects. This has led to the suggestion that rabbit γ-globulin is formed of two pieces with very similar structure joined to a third piece of quite different character.

I wish to thank Dr J. H. Humphrey for the gift of goat antiserum and advice on the immunization of rats, Mr E. E. McDermott and Dr J. Pace for their invaluable assistance in carrying out the amino acid analysis, Dr H. R. Perkins for the carbohydrate analysis and Mr M. Young for the photographs of the crystals. I also wish to thank Miss Hilary Morgan for her excellent technical assistance.

REFERENCES

Askonas, B. A., Humphrey, J. H. & Porter, R. R. (1956). *Biochem. J.* **63**, 412.
Boas, N. F. (1953). *J. biol. Chem.* **204**, 553.
Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
Freund, J. & McDermott, K. (1942). *Proc. Soc. exp. Biol., N.Y.*, **49**, 548.
Kabat, E. A. (1956). *J. Immunol.* **77**, 377.
Kabat, E. A. & Mayer, M. M. (1948). *Experimental Immunochemistry*, p. 59. Springfield, Illinois: C. C. Thomas.
Kekwick, R. A. (1940). *Biochem. J.* **34**, 1248.
Kimmel, J. R. & Smith, E. L. (1954). *J. biol. Chem.* **207**, 515.
McDermott, E. E. & Pace, J. (1957). *Brit. J. Nutr.* **11**, 446.
McFadden, M. L. & Smith, E. L. (1955). *J. biol. Chem.* **214**, 185.
Mokrasch, L. C. (1954). *J. biol. Chem.* **208**, 55.
Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
Pauling, L. (1940). *J. Amer. chem. Soc.* **62**, 2643.
Petermann, M. L. (1942). *J. biol. Chem.* **144**, 607.
Petermann, M. L. (1946). *J. Amer. chem. Soc.* **68**, 106.
Petermann, M. L. & Pappenheimer, A. M., jun. (1941). *J. phys. Chem.* **45**, 1.
Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **77**, 751.
Porter, R. R. (1950*a*). *Biochem. J.* **46**, 473.
Porter, R. R. (1950*b*). *Biochem. J.* **46**, 479.
Porter, R. R. (1955). *Biochem. J.* **59**, 405.
Porter, R. R. (1957*a*). *Methods in Enzymology*, vol. 4, p. 221. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Porter, R. R. (1957*b*). *Biochem. J.* **66**, 677.
Porter, R. R. (1958*a*). *Nature, Lond.*, **182**, 670.
Porter, R. R. (1958*b*). *Folia biol., Prague*, **4**, 310.
Sanger, F., Thompson, E. O. P. & Kitai, R. (1955). *Biochem. J.* **59**, 509.
Smith, E. L., McFadden, M. L., Stockell, A. & Buettner-Janesch, V. (1955). *J. biol. Chem.* **214**, 197.
Sober, H. A., Gutter, F. J., Wyckoff, M. M. & Peterson, E. A. (1956). *J. Amer. chem. Soc.* **78**, 756.

ADDENDUM

Ultracentrifugal Examination of Digestion Products from Rabbit γ-Globulin

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Sedimentation-velocity experiments were carried out at 59 780 rev./min. in a Spinco model E ultracentrifuge, the technique and calculation of results being as described by Charlwood (1952, 1955). Molecular-weight determinations were by a method suggested originally by Archibald (1947), the procedure used being that of Charlwood (1957). For these determinations the ultracentrifuge was run at 8210 or 12 590 rev./min. according to the sample being studied. Protein concentrations were measured in terms of the refractive increment between the protein solution and buffer against which it had been dialysed until in equilibrium. The differential refractometer used was of the type introduced by Cecil & Ogston (1951). A cell with a Kel-F (polychlorotrifluoroethylene) plastic centrepiece was used for most experiments in acetate buffer. In all calculations the partial specific volume was taken arbitrarily as 0·735, because no experimental values were available.

When digests of the γ-globulin were dialysed against sodium phosphate buffer (pH 6·8; I 0·2), some material (later identified with fraction III) precipitated, but the soluble part, on examination at top speed in the ultracentrifuge, showed a single, fairly symmetrical peak of sedimentation coefficient about 3·5s. The results of some of the experiments made with the isolated fractions are shown in Table 1, together with the corresponding figures for untreated γ-globulin. In all cases, with a possible exception referred to below, preparations were monodisperse in the ultracentrifuge.

The sedimentation coefficients of all three fractions were approximately the same ($\sim$3·5s) at the concentrations used in most of the experiments (0·65–0·82 %), and measurements made at lower concentrations, but not given in Table 1, showed that the concentration-dependence was small (not more than 3–4 % for a change in protein concentration of 1 %, w/v). Molecular weights measured

Table 1. *Molecular weights of digestion products from rabbit γ-globulins*

Rabbit γ-globulin was prepared by sodium sulphate fractionation of serum (Kekwick, 1940).

Source	Sample	Buffer	Refractive increment of protein solution	$S_{20,w}$	Molecular wt. ± S.D.
Rabbit γ-globulin (batch 1)	Whole globulin	Phosphate (pH 6·8; *I* 0·2)	0·00149	6·50 s	187 600 ± 1400
Rabbit γ-globulin (batch 2)	Fraction I	Phosphate (pH 6·8; *I* 0·2)	0·00147	3·59 s	49 600 ± 1800
	Fraction II	Phosphate (pH 6·8; *I* 0·2)	0·00138	3·55 s	52 600 ± 2600
	Fraction III	Acetate (pH 4·0; *I* 0·2)	0·00149	3·40 s	80 200 ± 9100
Rabbit γ-globulin (batch 3)	Fraction I	Phosphate (pH 6·8; *I* 0·2)	0·00132	—	50 400 ± 2000
	Fraction II	Phosphate (pH 6·8; *I* 0·2)	0·00118	—	55 700 ± 2000
	Fraction III	Acetate (pH 4·5; *I* 0·2)	0·00133	—	80 200 ± 1600

at the finite concentrations indicated should therefore not differ greatly from values which would be obtained by extrapolation to infinite dilution.

The molecular weights quoted in Table 1 were each derived by taking the mean value calculated from five photographic exposures made at 8- or 16-min. intervals. The standard deviations of individual values about the corresponding mean are given in the last column as a measure of internal consistency. Comparisons between means must in addition take into account the uncertainty residing in refractometer measurements, which amounted to about ±0·6 %, but which could affect a molecular weight to the extent of about ±0·9 %. Agreement between different measurements on corresponding fractions is considered to be reasonably good when allowance is made for these sources of variation. The only unsatisfactory set of measurements in Table 1 refers to fraction III at pH 4·0, where very erratic results were obtained, with some suggestion of a downward trend with increasing time. Moreover, there was a detectable asymmetry of the peak obtained in the ultracentrifuge at high speed, although the sedimentation coefficient of the maximum ordinate (3·40 s) was lower than for similar preparations at pH 4·5, which gave about 3·7 s. It was concluded that complications, which may include some aggregation and some molecular-shape change, make pH 4·0 unsuitable for investigation of fraction III.

The sum of the molecular weights of the three fractions is in good agreement with the molecular weight of whole γ-globulin, the figure for which corresponds fairly well with that most commonly accepted (170 000; see Porter, 1959).

REFERENCES

Archibald, W. J. (1947). *J. phys. Chem.* **51**, 1204.

Cecil, R. & Ogston, A. G. (1951). *J. sci. Instrum.* **28**, 253.

Charlwood, P. A. (1952). *Biochem. J.* **51**, 113.

Charlwood, P. A. (1955). *Canad. J. Chem.* **33**, 1043.

Charlwood, P. A. (1957). *Trans. Faraday Soc.* **53**, 871.

Kekwick, R. A. (1940). *Biochem. J.* **34**, 1248.

Porter, R. R. (1959). In *The Plasma Proteins*. Ed. by Putnam, F. W. New York: Academic Press Inc.

The *N*-Acetylation and Estimation of Hexosamines

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(*Received* 5 *March* 1959)

N-Acetylglucosamine is usually determined by Morgan & Elson (1934) colour reaction, as modified by Aminoff, Morgan & Watkins (1952). A more recent modification by Reissig, Strominger & Leloir (1955) is greatly superior on grounds of sensitivity, convenience and reproducibility, and in addition is unaffected by small quantities of acid or alkali in the test sample. Glucosamine in relatively large amounts does not interfere in the Morgan–Elson reaction.

The standard procedure for the estimation of glucosamine calls for preliminary treatment with alkaline acetylacetone (Elson & Morgan, 1933; Rondle & Morgan, 1955); under the usual conditions *N*-acetylglucosamine interferes to some extent. Interference by other tissue constituents is greater than in the *N*-acetylglucosamine determination. Conversion of glucosamine into *N*-acetylglucosamine as a prelude to assay by the Morgan–Elson reaction offers certain advantages

(Smithies, 1953; Pontis, 1955; Roseman & Daffner, 1956), not least that *N*-acetylglucosamine can be distinguished from *N*-acetylgalactosamine by the intensity of the colour produced.

There is some confusion on the subject of *N*-acetylation of glucosamine (see Kent & Whitehouse, 1955). *N*-Acetylglucosamine was first prepared by treating the free base with acetic anhydride in methanol (Breuer, 1898), but because of the alleged instability of the base the usual method of preparation employs glucosamine hydrochloride suspended in methanol and a mixture of silver acetate and acetic anhydride (Zuckerkandl & Messiner-Klebermass, 1931; White, 1940).

Selective *N*-acetylation of galactosaminic acid can be accomplished by adding acetic anhydride to a solution in excess of aqueous alkali (Karrer & Mayer, 1937), and this reaction has been extended to glucosaminic acid (Findlay, Levvy & Marsh, 1958). It therefore appeared probable that acetic anhydride could be employed to *N*-acetylate glucosamine in aqueous solution in the absence of excess of alkali, there being no question of zwitterion formation. [In this connexion one should mention the use of keten for the selective acetylation of the amino group in glucosamine derivatives in aqueous solution (Neuberger & Pitt-Rivers, 1939; Weissmann & Meyer, 1954).]

Methods for the *N*-acetylation of glucosamine hydrochloride in aqueous solution with acetic anhydride have already been described: a mildly alkaline pH was maintained throughout, on the preparatory scale by adding a large quantity of a basic ion-exchange resin (Roseman & Ludowieg, 1954), and on the analytical scale with excess of sodium bicarbonate (Roseman & Daffner, 1956). It is not clear whether alkali is added to prevent *O*-acetylation, or to act as an 'acid acceptor' in hastening the reaction.

Glucosamine can be quantitatively converted into *N*-acetylglucosamine in a few minutes, simply by adding acetic anhydride to aqueous solutions of the amino sugar, which are more stable than is generally believed. When the hydrochloride is employed, sodium hydroxide is added on the preparatory scale, but this is unnecessary on the analytical scale, since the solution is made sufficiently alkaline during chromogen formation. The reaction mixture can be taken directly for analysis by the method of Reissig *et al.* (1955), and this procedure can be applied to galactosamine also.

EXPERIMENTAL AND RESULTS

Estimation of N*-acetylglucosamine*

It is not clear from the description of Reissig *et al.* (1955) whether the molarity of their 0·8 M-potassium tetraborate reagent refers to BO_2^{2-} or $B_4O_7^{2-}$, but from our own experiments it would appear to refer to the latter. They obtained optimum colour development when the final $B_4O_7^{2-}$ concentration during chromogen formation was 0·133 M, but as shown in Fig. 1 we required 0·29 M-$B_4O_7^{2-}$ at this stage. Stock solutions of potassium tetraborate were made 0·7 M, this being the limit of solubility at room temperature. The salt was prepared as described by Reissig *et al.* (1955), and the pH of the stock solution was 9·2. A commercial specimen of potassium tetraborate gave a considerably higher pH (about 9·8) and, even after pH adjustment with HCl, led to lower readings in the colour reaction.

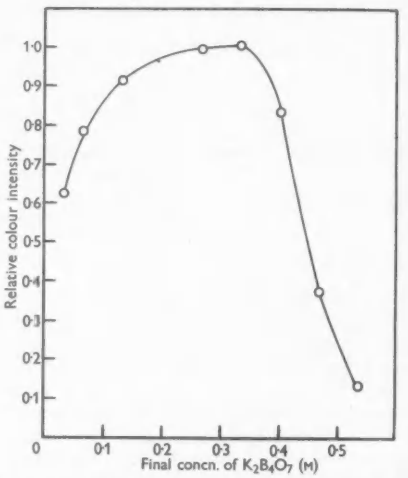

Fig. 1. Intensity of colour produced by *N*-acetylglucosamine (0·181 μmole) with varying concentrations of tetraborate during chromogen formation; the remainder of the procedure was as described in the text.

A standard curve was constructed by adding 0·5 ml. of 0·7 M-potassium tetraborate to 0·7 ml. of *N*-acetylglucosamine and following the procedure of Reissig *et al.* (1955): the results, which were perfectly reproducible from day to day, fell on the linear curve shown in Fig. 3 for glucosamine hydrochloride. Glucosamine in amounts up to 4·6 μmoles did not interfere in the colour reaction for *N*-acetylglucosamine.

Estimation of hexosamine

Standard procedure. The tube shown in Fig. 2 was convenient for carrying out the reaction. To 0·6 ml. of hexosamine solution (hydrochloride or free base) was added 0·1 ml. of 1·5% (v/v) acetic anhydride in acetone (prepared daily), followed by 0·5 ml. of 0·7 M-potassium tetraborate. A glass bulb was placed in the mouth of the tube, which was heated in a boiling-water bath for exactly 3 min. After cooling the tube, 6 ml. of the *p*-dimethylaminobenzaldehyde reagent of Reissig *et al.* (1955) was added. [AnalaR *p*-dimethylaminobenzaldehyde (British Drug Houses Ltd.) was entirely satisfactory.] The tube was closed with a glass stopper and the contents were mixed. Colour development was carried out for 20 min. at 37°, the glass stopper being momentarily released to equilibrate pressures. Colour

intensities were read almost immediately after cooling, with the Spekker photoelectric absorptiometer, Ilford no. 605 yellow–green filters (peak transmission at 545 mμ) and cells of 1 cm. length.

The total volume of the aqueous and acetone solutions was not appreciably altered after mixing, nor was there any change in volume after heating the mixture. Acetic anhydride in the quantity specified did not significantly alter the pH of the borate buffer. Pure *N*-acetylglucosamine was unaffected by the acetylation procedure.

Calibration curves for glucosamine hydrochloride and galactosamine hydrochloride are shown in Fig. 3. The specimen of galactosamine hydrochloride was presented by Dr P. G. Walker. It gave a colour intensity which was 35% of the value for glucosamine hydrochloride. This is in exact agreement with the figure obtained by Reissig *et al.* (1955) for *N*-acetylgalactosamine as compared with *N*-acetylglucosamine. Glucosamine itself gave readings identical with those for the hydrochloride.

The method is 2–3 times as sensitive as the acetylacetone method (Rondle & Morgan, 1955) for glucosamine; galactosamine gives the same intensity of colour as glucosamine in the latter procedure.

Stability of aqueous acetic anhydride. Fig. 4 shows the loss in efficacy of the standard acetic anhydride reagent in contact with water and glucosamine hydrochloride solutions. The acetone solution (0·1 ml.) was measured into 0·4 ml. of water and after varying periods 0·2 ml. of glucosamine hydrochloride solution (0·186 μmole) was added. After 1 or 15 min. the borate buffer was added and colour development was proceeded with in the usual way. It is evident that the interval before addition of borate should not exceed 15 min. There was a steep fall in the efficiency of the *N*-acetylation when the period of contact of glucosamine or glucosamine hydrochloride with acetic anhydride exceeded 20 min. before addition of borate.

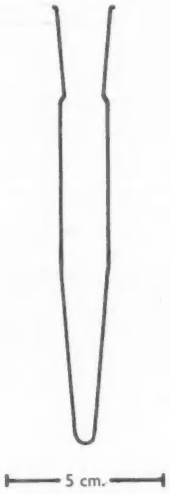

Fig. 2. Reaction tube for complete estimation, with B14 socket.

Stability of glucosamine base in aqueous solution. Very dilute solutions of glucosamine (0·93 mM, natural pH 8·5) were stable for several days at room temperature, as tested by the colour reaction, and could be maintained at 100° for 3 min. without decomposition (Table 1). More concentrated solutions (0·46 M, natural pH 9·5) showed no loss after 3 hr. and only 6% after 24 hr., but were rapidly destroyed at 100°. The more dilute solution, on being brought to pH 9·5 with NaOH, assumed the stability characteristics of the more concentrated solution.

Fig. 5 shows the rapid decay of a dilute solution of glucosamine hydrochloride in the presence of the borate buffer at room temperature before adding the acetic anhydride reagent: *N*-acetylglucosamine was completely stable under these conditions for 5 hr.

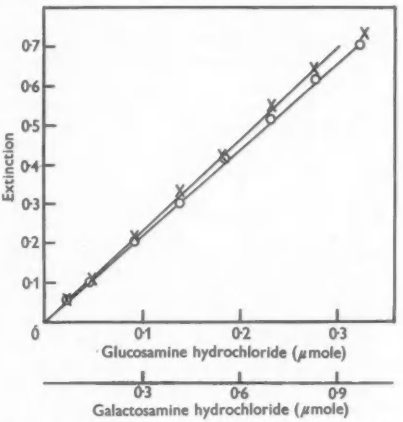

Fig. 3. Calibration curves for glucosamine hydrochloride (○) and galactosamine hydrochloride (×).

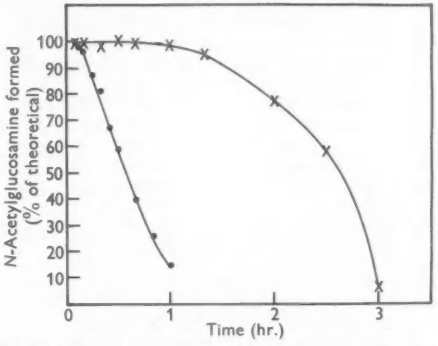

Fig. 4. Decay of acetic anhydride in aqueous solution as measured by the formation of *N*-acetylglucosamine; glucosamine hydrochloride was added after the intervals shown, and estimation proceeded with as described in the text. Borate buffer was added 1 min. (×) and 15 min. (●) after the glucosamine hydrochloride.

Table 1. *Stability of glucosamine base in aqueous solution*

NaOH was used for pH adjustment.

Concn. of glucosamine	pH	Temp.	Time	*N*-Acetylglucosamine (% of theoretical)
0·93 mM	8·5	Room temp.	4 days	98
		100°	3 min.	97
	9·5	Room temp.	3 hr.	98
			1 day	91
		100°	3 min.	52
	10·5	Room temp.	3 min.	98
0·46 M	9·5	Room temp.	3 hr.	100
			1 day	94
		100°	3 min.	68
	10·5	Room temp.	3 min.	98

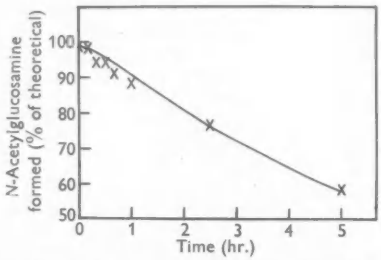

Fig. 5. Decay of glucosamine hydrochloride (0·186 μmole in 1·1 ml.) in the presence of the borate buffer. The acetic anhydride reagent was added after the intervals shown, and the tubes were then heated for chromogen development in the usual way.

Effect of varying the acetic anhydride concentration in the presence and absence of alkali. Fig. 6 shows the effect of varying the amount of acetic anhydride added during the standard assay procedure. The use of free glucosamine instead of the hydrochloride gave identical results. Addition of NaOH in low concentration did not diminish the amount of acetic anhydride required for complete development of colour (Fig. 7). Although esterification was incomplete, there was, however, greater acetylation of the amino group with low concentrations of acetic anhydride when alkali was added. Excess of NaOH relative to the acetic anhydride prevented the acetylation.

Effect of varying the pH *of the borate buffer.* Solutions of glucosamine hydrochloride were treated with acetic anhydride in the usual way, and then, after 1 min., with borate buffer of varying pH: 30 min. later the tubes were heated for chromogen formation. As shown in Table 2, replacing the standard borate buffer by one at pH 8 did not affect the result, but the use of a buffer at pH 10 led to much less development of colour. If the tubes were brought to pH 9·1 with NaOH or HCl immediately before heating, the same colour was seen in all. In other words, acetylation proceeded equally well at all three pH values, but chromogen formation was diminished at pH 10. Control experiments in which glucosamine hydrochloride was added last showed that no effective concentration of

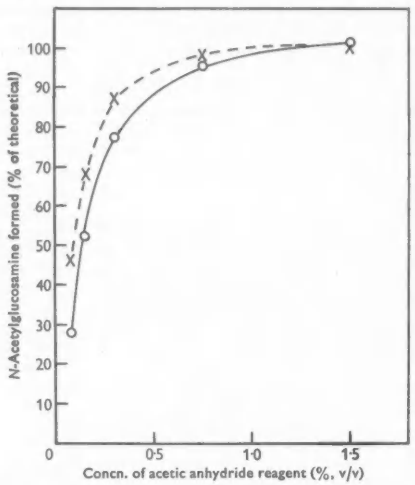

Fig. 6. Effect of varying the concentration of the acetic anhydride reagent in acetone; 0·1 ml. at the concentrations shown was added to glucosamine hydrochloride (0·186 μmole) in 0·6 ml. of water, and the estimation was continued as described in the text. ×, Borate added after 1 min.; ○, borate added after 15 min.

acetic anhydride remained after 30 min. contact with any of the borate buffers.

Similar experiments with *N*-acetylglucosamine (the addition of acetic anhydride being omitted) gave almost identical results. Reissig *et al.* (1955) found a sharp maximum in chromogen formation from *N*-acetylglucosamine in borate buffer at pH 9. Aminoff *et al.* (1952) found that borate buffers ranging from pH 9 to 11 were equally effective.

Preparation of N*-acetylglucosamine*

From glucosamine hydrochloride. At the amino sugar concentrations employed on the preparatory scale, it was possible to reduce the amount of acetic anhydride to only

a little more than the theoretical. Under these conditions, it was necessary to add NaOH to the hydrochloride. The effect of varying the amount of NaOH is shown in Fig. 8. For the purposes of isolation, 1–1·5 equiv. of NaOH was employed.

The preparation was carried out as follows. Glucosamine hydrochloride (10 g.) was dissolved at room temperature in water (60 ml.) containing 1 equiv. of NaOH. Acetic anhydride (7·5 ml.) was added, and after standing for 20 min. with occasional shaking the mixture was evaporated to dryness under reduced pressure. The white, crystalline residue was taken up in hot absolute methanol (400 ml.), the solution filtered, and the volume of filtrate reduced to 200 ml. Ether (600 ml.) was slowly added to precipitate a product (9·6 g.), m.p. 186–188°, containing Cl^- ion. It was dissolved in the minimum volume of hot water, 9 vol. of hot ethanol was added, and the hot mixture was filtered. The product that crystallized (6·8 g.), m.p. 193–195°, contained only a trace of Cl^- ion. Recrystallization from 75% (v/v) aqueous methanol yielded a chloride-free product (6·0 g.), m.p. and mixed m.p. with an authentic specimen 198–200° (corr.), $[\alpha]_D^{23} + 73° \rightarrow +37°$ in water (*c*, 1) (Found: $CH_3 \cdot CO^-$, 18·8. Calc. for $C_8H_{15}O_6N$: $CH_3 \cdot CO^-$, 19·4%). A second crop (0·6 g.), m.p. 196–198°, was obtained from the mother liquor. The pure product gave a single spot on a paper chromatogram (solvent, butanol–pyridine–water; spray, alkaline silver nitrate). Passage through acid ion-exchange resins or mixtures of acid and basic resins did not elevate the m.p. Recent literature gives m.p.'s ranging from 196° to 210° for *N*-acetylglucosamine; these may reflect differing proportions of the α- and β-anomers.

From glucosamine. Glucosamine was prepared from the hydrochloride by the simple and elegant method of Breuer (1898). The product melted at 116° (corr.) and was evidently largely in the β-form (Westphal & Holzmann, 1942). It was quite stable if stored dry in the dark.

The base (2·5 g.) in water (17·5 ml.) was treated with acetic anhydride (2·25 ml.), and the product was precipitated from methanol with ether as described above (2·31 g., m.p. 193–195°) and crystallized from 75% aqueous methanol. Yield 2·13 g., m.p. 198–200° (corr.).

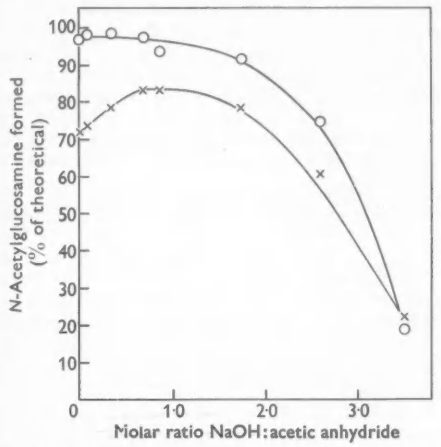

Fig. 7. Effect of adding varying proportions of NaOH during the acetylation of glucosamine hydrochloride (0·186 μmole). ○, Concentration of acetic anhydride reagent 0·75% (v/v) (8 μmoles employed); ×, concentration of acetic anhydride reagent 0·15% (v/v) (1·6 μmoles employed). Borate was added 1 min. after acetic anhydride.

Table 2. *Effect of varying the* pH *of the borate buffer during acetylation and chromogen formation*

Glucosamine hydrochloride (0·186 μmole) was treated with 1·5% (v/v) acetic anhydride solution and 0·7 M-$B_4O_7^{2-}$ solution in the usual way, but the borate solution was of varying pH. An interval of 30 min. was allowed to elapse before heating, and in the second experiment all the tubes were brought to the same pH (9·1) at this point.

Expt.	pH of borate buffer	Colour intensity (% of theoretical)
1	8·1	97
	9·2	97
	10·0	23
2	8·1	98
	9·2	99
	10·0	99

DISCUSSION

It would appear that fairly concentrated aqueous solutions of glucosamine are sufficiently alkaline for *N*-acetylation with acetic anhydride to proceed in

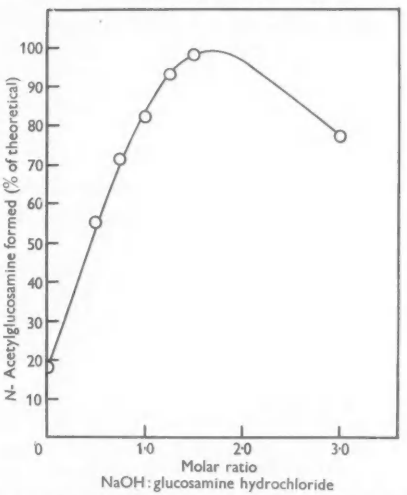

Fig. 8. Effect of adding varying proportions of NaOH during the acetylation of glucosamine hydrochloride on the preparatory scale; 100 mg. of glucosamine hydrochloride in 1·0 ml. of aqueous alkali was treated with 0·075 ml. of acetic anhydride (1·7 mol. prop.) for 15 min., and *N*-acetylglucosamine determined as described in the text.

the absence of added alkali. With more dilute solutions and with solutions of the hydrochloride it is necessary to add alkali. For purposes of estimation by the colorimetric method of Reissig *et al.* (1955) for *N*-acetylglucosamine, the borate buffer employed for chromogen formation is sufficiently alkaline. If present in molar excess, relative to acetic anhydride, sodium hydroxide prevents acetylation by competition with the amino group.

Acetylation of the amino group in glucosamine with acetic anhydride in aqueous solution seems to be almost instantaneous. In their method for the estimation of hexosamine, based on this reaction, Roseman & Daffner (1956) employ sodium bicarbonate as the catalyst. After 10 min. at room temperature, excess of acetic anhydride is destroyed by heating to 100°. Sodium carbonate is then added for the determination of *N*-acetylglucosamine by the procedure of Aminoff *et al.* (1952).

SUMMARY

1. Glucosamine and galactosamine can be determined by the method of Reissig, *et al.* (1955) for the *N*-acetylated hexosamines by preliminary treatment in aqueous solution with acetic anhydride. The borate buffer employed for chromogen formation catalyses the *N*-acetylation of the hexosamines.

2. *N*-Acetylglucosamine can be prepared from glucosamine base by treatment of aqueous solutions with acetic anhydride. If glucosamine hydrochloride is employed alkali must also be added.

REFERENCES

Aminoff, D., Morgan, W. T. J. & Watkins, W. M. (1952). *Biochem. J.* **51**, 379.
Breuer, R. (1898). *Ber. dtsch. chem. Ges.* **31**, 2193.
Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
Findlay, J., Levvy, G. A. & Marsh, C. A. (1958). *Biochem. J.* **69**, 467.
Karrer, P. & Mayer, J. (1937). *Helv. chim. acta*, **20**, 407.
Kent, P. W. & Whitehouse, M. W. (1955). *Biochemistry of the Aminosugars*, p. 215. London: Butterworth.
Morgan, W. T. J. & Elson, L. A. (1934). *Biochem. J.* **28**, 988.
Neuberger, A. & Pitt-Rivers, R. V. (1939). *J. chem. Soc.* p. 122.
Pontis, H. G. (1955). *J. biol. Chem.* **216**, 195.
Reissig, J. L., Strominger, J. L. & Leloir, L. F. (1955). *J. biol. Chem.* **217**, 959.
Rondle, C. J. M. & Morgan, W. T. J. (1955). *Biochem. J.* **61**, 586.
Roseman, S. & Daffner, I. (1956). *Analyt. Chem.* **28**, 1743.
Roseman, S. & Ludowieg, J. (1954). *J. Amer. chem. Soc.* **76**, 301.
Smithies, W. R. (1953). *Biochem. J.* **53**, xxix.
Weissmann, B. & Meyer, K. (1954). *J. Amer. chem. Soc.* **76**, 1753.
Westphal, O. & Holzmann, H. (1942). *Ber. dtsch. chem. Ges.* **75**, 1274.
White, T. (1940). *J. chem. Soc.* p. 428.
Zuckerkandl, F. & Messiner-Klebermass, L. (1931). *Biochem. Z.* **236**, 19.

Studies on the Phosphoproteins of Brain: the Intracellular Localization in Brain of a Phosphoprotein Involved in the Metabolic Response of Cortical Slices to Electrical Stimulation

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The application of electrical pulses for 10 sec. to slices of cerebral cortex which had metabolized radioactive phosphate for a few minutes resulted in marked changes in the distribution of radioactivity in phosphocreatine, adenosine triphosphate and in the phosphoprotein fraction (Heald, 1956, 1957). Under these conditions, the specific radioactivity of phosphocreatine and the γ-phosphorus of adenosine triphosphate decreased (Heald, 1956), whereas that of the phosphoprotein phosphorus increased (Heald, 1957). Subsequently it was found that acid hydrolysis of the radioactive phosphoprotein fraction yielded only radioactive phosphorylserine (Heald, 1958).

Sequences illustrating ways in which such changes in phosphates may be induced by electrical pulses have been outlined by McIlwain & Gore (1953) and Heald (1956). Briefly, pulses are considered to alter structures involved in the transport of ions from one membrane surface to another, and the accompanying changes induced in the phosphates are considered to reflect the increased

energy expenditure necessary to restore the disturbed ionic balance. Present evidence indicates that phosphoprotein is more closely associated with the structures changed by pulses than is a phosphate such as adenosine triphosphate.

In such sequences phosphoprotein is assigned a role either similar to that of a phosphotransferase or as an intermediate in the transfer of energy-rich phosphorus, and not that of a simple acceptor of phosphate in a system in which the activity of the enzymic phosphorylating mechanisms has been increased by the experimental procedure. This view is supported by the finding that there is no change in the total quantity of phosphoprotein phosphorus accompanying the increase in specific radioactivity resulting from the application of pulses (Heald, 1957).

Enzymes known to yield phosphorylserine on acid hydrolysis, and to become phosphorylated during their action, are yeast hexokinase (Agren & Engstrom, 1956) and liver phosphorylase (Rall, Sutherland & Wosilait, 1956; Wosilait, 1958). Other phosphorylated proteins which are enzymes include pepsin (see Neurath, 1957) and phosphoglucomutase (Sidbury & Najjar, 1957). Suggestions that the phosphoproteins of brain may be of this type have been made by Engelhardt & Lissovskaya (1953) and Rossiter (1955).

On the other hand, the phosphoprotein increasing in radioactivity as a result of application of pulses to cerebral tissues may be non-enzymic and occupy a position as a source of energy-rich phosphorus in the brain. In this respect it could be analogous to phosvitin, which has been shown to be capable of phosphorylating adenosine diphosphate to the triphosphate in the presence of a yeast phosphokinase, and of being phosphorylated in the reverse reaction (Rabinowitz & Lipmann, 1958). This is considered to be a more probable role of phosphoprotein in brain, for the quantities of phosphoprotein phosphorus contributed by enzymes such as those described above, assuming a similarity with brain enzymes, can be calculated to be not more than 1–2% of the total quantity of phosphoprotein phosphorus found.

In either case the knowledge that the phosphoprotein fraction exhibits an increased turnover of phosphorus in response to a very brief application of pulses to the tissue makes it of interest to examine its nature and metabolism. At the start of this work no evidence was available to indicate that the phosphorus of the phosphoprotein fraction was derived from other than a single substance. As a preliminary to the attempted isolation of the phosphoprotein it was therefore considered desirable to investigate this point, as far as possible, and to discover the subcellular fraction containing the phosphoprotein with a view to obtaining a starting material reasonably free from other contaminants.

The results described below show that brain contains at least two phosphoprotein fractions, in only one of which the phosphorus increases in specific radioactivity when electrical pulses are applied to the intact tissue.

MATERIALS AND METHODS

Tissues and media. Slices of guinea-pig cerebral cortex were prepared as described by McIlwain (1951) and incubated in medium buffered with 2-amino-2-hydroxymethylpropane-1:3-diol, from which added phosphate was absent (Heald, 1956). Slices of 100–150 mg. wet wt. were incubated in 3·5 ml. of medium in conventional Warburg manometric flasks under an atmosphere of oxygen at 37·5°, in the presence of 10–20 μc of carrier-free radioactive orthophosphate (The Radiochemical Centre, Amersham, Bucks). Incubations were usually for 60–90 min., at the end of which the contents of the manometric flasks were either tipped into an open dish and the slices rapidly transferred to 10 vol. of ice-cold sucrose solution, or, more frequently, tipped into a large tube cooled in ice, and briefly centrifuged and the tissue residue was dispersed in 10 vol. of sucrose solution. The usual medium for preparing the dispersions was sucrose (0·25 M) containing ethylenediaminetetra-acetate (EDTA) (0·5 mM), adjusted to pH 7·4 with NaOH solution. In experiments *in vivo* the radioactive phosphate was injected intracisternally by a technique similar to that of Strickland (1952). The animals were lightly anaesthetized with ether before puncture of the cisterna magna; about 0·2 ml. of fluid was withdrawn and was replaced by a solution of radioactive phosphate injected through the same needle. After 3 hr. the animal was killed, the brain removed and the cortex, together with some underlying white matter, was weighed and dispersed in 10 vol. of sucrose–EDTA. All subsequent operations with sucrose suspensions were at 0–2°.

For short-term experiments, in which electrical pulses were applied for brief periods, the apparatus and techniques have been described (Heald, 1956). In experiments *in vivo* in which animals were frozen in liquid nitrogen before removal of the brain, the procedure was similar to that described by Dawson & Richter (1950) and Kratzing & Narayanaswami (1953).

Dispersion of the tissue. Since the biochemical integrity of subcellular particles is affected by the method of producing the initial dispersions of the tissue (Aldridge, 1957), in the present experiments all dispersions were made with a polythene pestle constructed according to the method of Kamphausen & Morton (1956). Such pestles fitted the tubes tightly and produced excellent dispersions, in which few intact cells were visible when viewed under phase contrast but in which the nuclei were maintained largely intact. This was taken to indicate that little general mechanical disruption of the particles had taken place during the initial stage of dispersion of the tissue.

Fractionation of dispersions. The sucrose dispersions were initially fractionated by differential centrifuging mainly according to the procedure of Brody & Bain (1952). Dispersions were first centrifuged for 10 min. at 1500 g to obtain the 'nuclear fraction', which was washed by resuspending it in sucrose–EDTA and recentrifuging for 10 min. at 1500 g. The supernatants were centrifuged either at 104 000 g for 30 min., to obtain a composite

fraction of mitochondria and microsomes, or else centrifuged first at 12 000 *g* for 25 min., to obtain the mitochondrial fraction, and then at 104 000 *g* for 30 min. to yield the microsomal fraction and a clear supernatant. The nuclear fraction was further subfractionated by centrifuging in various sucrose density gradients as indicated in the figures and text. This procedure was carried out in the S.W. 39 head of the Spinco ultracentrifuge. Other centrifugings were performed in the no. 40 head of this instrument. All fractions were treated with trichloroacetic acid to a concentration of 10% (w/v) before obtaining the phosphoprotein.

Succinic dehydrogenase. This was determined in various subcellular fractions by the method of Schneider & Potter (1943).

Total nitrogen. This was determined after digestion of the fraction by the semi-micro procedure and catalyst of Chibnall, Rees & Williams (1943). The final distillation was carried out in the Markham (1942) still, with boric acid solution (Conway, 1947) as the trapping agent.

Protein. This was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with amorphous albumin fraction IV (L. Light and Co. Ltd.) as a standard.

Phosphoprotein. The quantitative determination of phosphoprotein as inorganic phosphorus and determinations of its specific radioactivity were performed as described previously (Heald, 1957), by the method for preparing the phosphoprotein fraction described in that paper. Extensive use has also been made of the finding (Heald, 1958) that the phosphoprotein fraction sought yielded phosphorylserine upon acid hydrolysis. Preparations of crude phosphoprotein obtained from tissue slices which had been allowed to metabolize radioactive phosphate were hydrolysed for 8–10 hr. at 100° in 2N-HCl. The hydrolysates were freeze-dried and the residues, in 0·05N-HCl, were fractionated upon a column of Dowex-50 as described by Schaffer, May & Summerson (1953). Inactive phosphorylserine was added as a carrier. Since the position of phosphorylserine in eluates from such a column was highly reproducible, and it appeared to be the only major radioactive peak other than inorganic phosphate, counts due to the presence of radioactive phosphorylserine could be readily detected. In any one experiment where the distribution of the phosphoprotein in different fractions was being investigated, all hydrolyses were performed at the same time in the same heating bath. Under these conditions it was assumed that the partial hydrolysis of phosphorylserine would be the same in all fractions. Within such an experiment the results obtained are thus comparable with one another. Comparison between different experiments was made on a percentage basis, since in different experiments differing tissue weights and quantities of radioactive phosphate were used.

Acid and alkaline phosphatase activity. These were determined by the method of King & Armstrong, as described by Harrison (1947), disodium phenylphosphate being used as substrate.

RESULTS

In initial experiments tissues were dispersed in sucrose–EDTA solutions, the dispersions were centrifuged to yield the supernatant and particulate fractions and the radioactivity of the phos-

Table 1. *Distribution of phosphoprotein between the particulate and supernatant fractions of guinea-pig-brain dispersions* in vitro *and* in vivo

Tissues were dispersed in 0·25M-sucrose–0·5 mM-EDTA. Phosphoprotein was determined as radioactive phosphorylserine. For details see Materials and Methods section. Values are given ± S.D. Figures in parentheses are the number of determinations.

Tissue	Fraction	Nitrogen (mg./g. wet wt.)	Nitrogen (% of total)	Protein (mg./g. wet wt.)	Protein (% of total)	Phosphoprotein (% of total)	Phosphoprotein (%) Nitrogen (%)	Phosphoprotein (%) Protein (%)
Slices incubated *in vitro*	Insoluble	13·87 ± 1 (4)	64·6 ± 1·5	57·3 ± 4 (4)	78·1 ± 5·1	87·2 ± 2·7 (10)	1·35	1·12
	Soluble	7·2 ± 2 (4)	35·4 ± 5·6	21·9 ± 5 (3)	21·9 ± 2·20	12·8 ± 2·4 (10)	0·36	0·58
Cortex, *in vivo*	Insoluble	12·39 ± 0·2 (3)	62·1 ± 1·6	49·3 ± 3·5 (6)	60·1 ± 11·2	44·0 ± 5·6 (6)	0·66	0·68
	Soluble	7·80 ± 1·4 (3)	37·9 ± 3·8	27·2 ± 4·6 (6)	39·2 ± 6·0	56·0 ± 5·5 (6)	1·56	1·48

phorylserine derived from the phosphoprotein fraction was determined. Experiments were performed both with slices of tissue which had been incubated in medium containing radioactive phosphorus and with brain cortex from guinea pigs which had been injected, *in vivo*, intracisternally with radioactive phosphorus some 2–3 hr. before removal of the brain. The experiments *in vivo* were originally included as a check upon the distribution of phosphoprotein found in slices *in vitro*, for it was thought that incubation of tissues in medium might have caused changes in the slices, sufficient to alter the distribution of the phosphoprotein. The results are presented in Table 1.

With slices most of the radioactivity arising from the phosphoprotein was present in the particulate fraction, being concentrated in terms of nitrogen or protein some three to four times that in the soluble fraction. *In vivo* the reverse situation obtained, the radioactivity from phosphoprotein being highest in the supernatant fraction, where it was concentrated in terms of nitrogen or protein some two times that in the insoluble fraction. During experiments *in vivo*, removal and weighing of the brain occupied some 4–5 min. before it was dispersed, as opposed to 10 sec. for a similar transfer of slices from the incubation medium to the sucrose solution. It was therefore considered possible that some rapid enzymic action after death might be releasing phosphoprotein from a more firmly bound form, which was later reformed during metabolism *in vitro*. Experiments were carried out in which the brain was frozen *in vivo* in liquid nitrogen before being chiselled out and fractionated. This procedure had little effect upon the distribution of radioactivity (Table 2). It was also found that dispersion of tissues, from experiments *in vivo*, in sucrose solution containing 0·1 M-KCl, did not alter the distribution of radioactivity sufficiently to suggest that the presence of medium, carried with the slices during transfer from the incubation medium to sucrose solution, caused enough agglutination of the soluble phosphoprotein to account for the distribution found with slices incubated *in vitro*.

Conversely, with brain slices, freezing the tissue before dispersion, or applying brief electrical pulses to simulate the stimulus to the brain upon death, was without appreciable effect upon the distribution of radioactive phosphoprotein (Table 3). The

Table 2. *Distribution of phosphoprotein between the soluble and particulate fractions of dispersions of guinea-pig cerebral cortex obtained* in vivo

Animals were injected intracisternally with radioactive phosphorus 2–3 hr. before death. Tissues were dispersed in 0·25 M-sucrose with additions detailed below. Phosphoprotein was determined as radioactive phosphorylserine.

Expt. no.	Treatment of tissue	Phosphoprotein (counts/min.)		Phosphoprotein (% of total)	
		Residue	Supernatant	Residue	Supernatant
1	Brain removed and dispersed in 0·25 M-sucrose containing EDTA (5 mM)	2900	4232	41·2	58·8
2	Brain removed and dispersed:				
	(*a*) in 0·25 M-sucrose	2197	4183	34·3	65·7
	(*b*) in 0·25 M-sucrose + 0·1 M-KCl	1260	960	56·5	43·5
3	Brains frozen *in vivo* in liquid nitrogen	810	1640	33·0	67·0
		1430	3097	33·2	66·8
	Dispersed in 0·25 M-sucrose	460	611	43·0	57·0

Table 3. *Effect of different treatment of slices of guinea-pig-brain cortex upon the distribution of phosphoprotein between the soluble and particulate fractions*

Slices were incubated in medium containing radioactive orthophosphate and were dispersed in 0·25 M-sucrose containing additions as stated. Where electrical pulses were passed these were condenser pulses, 15 v peak potential, duration 0·4–0·5 msec. Phosphoprotein was determined as radioactive phosphorylserine.

Expt. no.	Treatment of tissue	Radioactivity in phosphoprotein (counts/min.)		Radioactivity in phosphoprotein (% of total)	
		Residue	Supernatant	Residue	Supernatant
1	Dispersed in sucrose	7757	1109	87·5	12·5
2	Dispersed in sucrose and EDTA (50 mM)	3877	623	85·0	15·0
3	Dispersed in water	1458	1516	49·0	51·0
4	Electrical pulses applied for 10 sec. before dispersion	3549	320	92·0	8·0
5	Slices frozen in solid CO_2 for 5 min. before dispersion	2260	657	78·0	22·0

Table 4. *Effect of incubating guinea-pig-brain slices in medium upon the quantities of phosphoprotein present in the soluble and particulate fractions of the tissue*

Slices were incubated in medium for 30 min. before being removed and dispersed in 0·25 M-sucrose–0·5 mM-EDTA. Analyses were as described in the Materials and Methods section. Values for phosphoprotein are in μmoles of P/g. wet wt. of tissue. Values for protein are in mg./g. wet wt. of tissue. Figures in parentheses indicate the number of experiments. Protein values are averages of two determinations.

	Before incubation		After incubation		Change	
Fraction	Phosphoprotein	Protein	Phosphoprotein	Protein	Phosphoprotein	Protein
Particulate	0·57±0·1 (4)	91	0·48±0·07 (4)	99	−0·09±0·01 (4)	+8
Soluble	0·40±0·01 (4)	42·1	0·22±0·01 (4)	25·6	−0·18±0·01 (4)	−16·5
Medium	0·0	0·0	0·17±0·008 (4)	21·5	+0·17±0·008 (4)	+21·5
Total	0·97±0·12 (4)	133·1	0·90±0·10 (4)	146·1	−0·12±0·07 (4)	+13

inclusion of EDTA (50 mM) in the sucrose solution likewise did not result in more of the radioactivity passing to the solution, indicating again that the greater concentration of radioactive phosphoprotein in the particulate fraction was not due simply to a precipitation by salts carried from the incubation medium. Even when the tissues were dispersed in water no more than 50 % of the total radioactive phosphoprotein of the slice was found in the supernatant fraction.

These results suggested that during incubation there was either a loss of phosphoprotein from the slice to the medium, or that the incorporation of radioactivity into the supernatant phosphoprotein was poor. Phosphoprotein and total protein were estimated in the supernatant and particulate fractions of cortical slices cut and weighed before incubation and in slices from the same brain after incubation. Similar determinations were also performed upon the incubation medium. There was a loss of some 50 % of the phosphoprotein phosphorus and of protein from the supernatant fraction to the medium during incubation, whereas little change occurred in the quantity of these substances in the particulate fraction (Table 4). This last result confirmed that incubation in medium did not result in an increased quantity of phosphoprotein from the supernatant being precipitated to form part of the particulate fraction. To determine whether the two fractions incorporated phosphate at different rates, slices of brain cortex were incubated with radioactive phosphate for different times and the specific radioactivities of the phosphoprotein present in the two fractions were determined. The result (Table 5) showed that the specific radioactivity of the phosphoprotein in the supernatant fraction was several times lower than that in the particulate fraction. Thus the phosphoprotein in the supernatant fraction incorporated radioactive phosphorus to a lesser degree than that in the particulate fraction. The differences in the distribution of radioactive phosphoprotein in brain tissues *in vivo* and slices *in vitro* is therefore partly attributable to a loss of part of the fraction soluble in aqueous sucrose to the medium during incubation of the slices and to a lowered rate of incorporation of phosphate into that remaining in the slices.

Table 5. *Incorporation of radioactive orthophosphate into the phosphoproteins of the soluble and particulate fractions of guinea-pig-brain slices*

Slices were incubated under oxygen, in medium containing carrier-free radioactive orthophosphate (10 μc/flask). After 15 or 30 min. the slices were removed, dispersed in 0·25 M-sucrose–0·5 mM-EDTA and the dispersion was centrifuged for 20 min. at 104 000 *g*. The residue and supernatant were treated to obtain the phosphoproteins as described in the Materials and Methods section.

Expt. no.	Period of incubation (min.)	Specific radioactivity (counts/min./μg. of phosphoprotein P)	
		Residue	Supernatant
1	15	958	218
	30	1700	245
2	15	716	143
	30	1748	505

Fractionation of the tissue into subcellular fractions. In view of the results described above, all further work was carried out with tissue slices incubated in medium containing radioactive phosphate. After incubation the slices were centrifuged from the medium and dispersed in aqueous sucrose. In each fraction, phosphoprotein was determined as radioactive phosphorylserine. Of the total radioactivity 66 % was present in the nuclear fraction, where its concentration was some two times that of other fractions (Fig. 1). The amount present in the mitochondrial fraction probably represented the degree of contamination with particles from the nuclear fraction.

To determine whether the nuclear fraction actually contained the phosphoprotein sought, tissues were incubated for a brief period of 3 min. in the presence of radioactive orthophosphate, and washed and reincubated in fresh medium for 2 min.

and then subjected to electrical pulses for 10 sec. This procedure is identical with that previously used to demonstrate the participation of phosphoproteins in the increased metabolic response of brain tissue to an electrical stimulus. After stimulation the tissues were rapidly transferred to ice-cold sucrose–EDTA solution, and dispersed, and the nuclear fraction separated. The major increase in specific radioactivity occurred in the phosphoprotein present in the nuclear fraction (Table 6), whereas a small and variable change took place in the phosphoprotein of the supernatant, which also contained the mitochondria and microsomes. It was clear that the phosphoprotein involved in the metabolic response to pulses resided largely in the nuclear fraction.

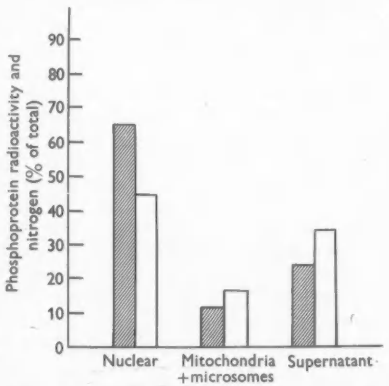

Fig. 1. Distribution of phosphoprotein radioactivity and nitrogen in subcellular fractions from guinea-pig-brain slices, after incubation in medium. The 'nuclear' and 'mitochondrial and microsomal' fractions were obtained by centrifuging dispersions, made in 0·25 M-sucrose–0·5 mM-EDTA, in integrated fields of 15×10 *g* min. and 9×10 *g* min. respectively. Radioactivity in phosphoprotein was determined as described in the Materials and Methods section. ▨, Phosphoprotein; □, nitrogen.

The nuclear fraction was subfractionated by equilibrium centrifuging in a sucrose density gradient (cf. Kuff & Schneider, 1954) in two stages. In the first sub-fractionation the nuclear fraction in 0·25 M-sucrose–EDTA was layered over M-sucrose and centrifuged to yield two main fractions. The top layer not entering M-sucrose consisted largely of nuclei and platelets together with some very small particles. The residue consisted of blood vessels, some platelets, myelin fragments and cell debris together with a large number of particles resembling mitochondria in size. This residue contained the bulk of the nitrogen [78·8 ± 2·2 (9) %] and phosphoprotein radioactivity [87·4 ± 3·3 (6) %] of the nuclear fraction, the ratio of the percentage of radioactivity to nitrogen being 1·10. The top layer contained 21·7 ± 1·8 (10) % of nitrogen and 13·0 ± 2·7 (6) % of the phosphoprotein, the ratio of phosphoprotein radioactivity to nitrogen being 0·6. Recoveries of both nitrogen and radioactive phosphoprotein were 100 %, showing that no loss had occurred during this stage of the fractionation procedure. The residue was resuspended in 0·25 M-sucrose and layered over a gradient consisting of three successive 1 ml. portions of 1·0 M-, 1·2 M- and 1·4 M-sucrose. The tubes were centrifuged and the various layers removed carefully with a fine Pasteur pipette. This procedure is presented in Fig. 2, together with the distribution of phosphoprotein radioactivity and nitrogen in the fractions obtained. Fraction R_2 contained the highest concentration of phosphoprotein and nitrogen, the ratio of the percentages of these two being 1·05; all the other fractions contained more nitrogen than phosphoprotein. The recovery of nitrogen in this step was 109 %, whereas the recovery of phosphoprotein was only 70 %, which suggests that some metabolism had occurred during the long periods of centrifuging.

In the above-described experiments measurement of the quantities of phosphoprotein had been made on the basis of the radioactivity of the phosphorylserine derived from the phosphoprotein

Table 6. *Effect of electrical pulses on the incorporation of radioactive phosphorus into the phosphoproteins of brain fractions*

Slices of guinea-pig-brain cortex were incubated and exposed to pulses under the conditions described by Heald (1957). Pulses were condenser pulses, 15 v peak potential, 0·4–0·5 msec. duration, applied for 10 sec. The nuclear fraction was separated from dispersions made in 0·25 M-sucrose–0·5 mM-EDTA by centrifuging (Brody & Bain, 1952). Phosphoprotein was determined as described in the Materials and Methods section.

	Radioactivity of the phosphoprotein (counts/min./μg. of P)					
	With pulses		Without pulses		Change with pulses	
Expt. no.	Nuclear	Supernatant	Nuclear	Supernatant	Nuclear	Supernatant
1	1770	417	922	445	+748	−45
2	2720	480	1580	362	+1140	+118

present. Since each fraction might have contained phosphoproteins of differing specific radioactivities, measurements of the total radioactivity could not strictly be taken as a measurement of quantity. The validity of this procedure was therefore examined by measuring the specific radioactivity of phosphoprotein phosphorus in the various fractions. Slices of cerebral cortex were incubated with radioactive inorganic phosphorus for different times before fractionation, since it was thought that such a procedure would emphasize whether the phosphoprotein in any one fraction incorporated radioactive phosphorus to a markedly greater or lesser extent than the others. The results are presented in Table 7. Comparison of specific radioactivities of the different fractions was made by relating them to that of fraction R_2. Although there was sometimes marked variation in any one experiment the averages of the sums of the ratios for each fraction did not differ significantly. Similar results were obtained if the comparison was made by relating the specific radioactivities to those of fraction R_3. The results are considered to show that the incorporation of radioactive phosphorus into the phosphoprotein of the individual particulate fractions proceeded to essentially the same extent, and thus the use of radioactivity as a measure of the quantity of phosphoprotein was a valid one. When viewed by phase-contrast microscopy, fraction R_1 consisted of extremely small particles together with some nuclei and platelets; R_2 consisted of mitochondria-like particles and was not homogeneous (particles of many different shapes being visible); R_3 was similar to R_2 except that the particles appeared to be larger, and R_4 contained cellular debris, myelin fragments and blood vessels together with a few small particles. Fraction R_2 was diffused on either side of the boundary between 1·0 M- and 1·2 M-sucrose layers, which suggested the possibility of further subfractionation. This was achieved by resuspension in 0·25 M-sucrose and layering of the suspension over 1 ml. portions of 1·0 M-, 1·1 M- and 1·2 M-sucrose. After centrifuging, the fractions were removed and analysed for phosphoprotein and nitrogen. The results, presented in Fig. 3, show that the fraction $R_{2(b)}$ contained the highest concentration of phos-

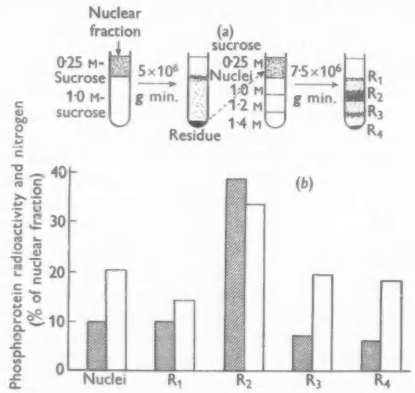

Fig. 2. Subfractionation, in a sucrose density gradient, of the 'nuclear' fraction (Fig. 1) obtained from guinea-pig-brain slices. (*a*) Details of concentrations of sucrose and centrifugal fields used; (*b*) the distribution of phosphoprotein radioactivity and nitrogen amongst the main fractions obtained. ▨, Phosphoprotein; □, nitrogen.

Table 7. *Comparison of the relative specific radioactivities of phosphoprotein in different subcellular fractions from cerebral cortex*

Slices were incubated in medium with different amounts of radioactive orthophosphate for varying times and were disintegrated and fractionated as described in the Materials and Methods section and in the text. Specific radioactivities were estimated after alkaline degradation of the phosphoprotein to inorganic phosphate as described in the text, with the exception of Expt. 7, in which fractions were acid-hydrolysed to yield phosphorylserine, which was separated on Dowex-50 and wet-ashed to yield inorganic phosphate.

Expt. no.	Period of incubation (min.)	Specific radioactivities of the phosphoprotein in the various fractions (counts/min./μg. of P)				Ratio: Specific radioactivity of fraction / Specific radioactivity of fraction R_2			
		Nuclei and R_1	R_2	R_3	R_4	Nuclei and R_1	R_2	R_3	R_4
1	3	—	75·5	75·3	65·5	—	1·0	1·0	0·86
2	3	—	1010	667	900	—	1·0	0·67	0·89
3	3	—	1080	904	815	—	1·0	0·84	0·75
4	10	103	200	246	Lost	0·52	1·0	1·17	—
5	10	87·5	72	66	Contaminated	1·22	1·0	0·62	—
6	20	241	475	345	Contaminated	0·51	1·0	0·73	—
7	30	515	422	396	Lost	1·21	1·0	0·94	—
Averages ± S.D.M.	—	—	—	—	—	0·86 ± 0·38	1·0	0·85 ± 0·19	0·83 ± 0·075

phoprotein with the fraction $R_{2(c)}$ containing a somewhat lower concentration. Here also, recoveries of nitrogen were 108%, whereas the recovery of phosphoprotein was only 80%. With phase-contrast microscopy, recognition of individual differences between the fractions was difficult; each fraction, however, appeared to be much more homogeneous in the type of particle present and was clearly different from the parent fraction R_2.

In Table 8 is presented a summary of the recoveries and distribution of phosphoprotein and nitrogen of the various fractions, compiled from the tables and figures previously given. The overall recovery of nitrogen was 119%, whereas the recovery of radioactive phosphorylserine was only 55%. These different recoveries are reflected in the low ratios of phosphorylserine to nitrogen in the last column of Table 8. However, since the degree of metabolism of phosphoprotein by the individual fractions is not known, no corrections can be made for such losses.

Succinic dehydrogenase and phosphatases in fractions R_1–R_4. Fractionation of dispersions of brain by the method of Brody & Bain (1952) is known to include some mitochondria in the nuclear fraction. This inclusion is increased when dispersions are made from slices of brain which have been previously incubated in medium. Thus the percentages of nitrogen of the dispersion found in the nuclear and mitochondrial fractions from brain dispersed immediately after removal from the animal were 27·4 and 34·6% respectively, and the percentages of succinic dehydrogenase in the fractions were 13 and 69% respectively. Similar fractions from slices incubated *in vitro* gave nitrogen distributions of 47·6 and 16·9% and a succinic dehydrogenase distribution of 52 and 34% respectively. It was therefore considered possible that the particles isolated could consist largely of mitochondria. The distribution of succinic dehydrogenase in the fractions R_1–R_4 is presented in Fig. 4 together with the corresponding figures for the distribution of phosphoprotein. The greatest activity was found in fractions R_3 and R_4, which contain the least phosphoprotein, whereas in R_1 no succinic dehydrogenase was found in any of three experiments. When a sample of mitochondria, prepared from guinea-pig brain immediately after

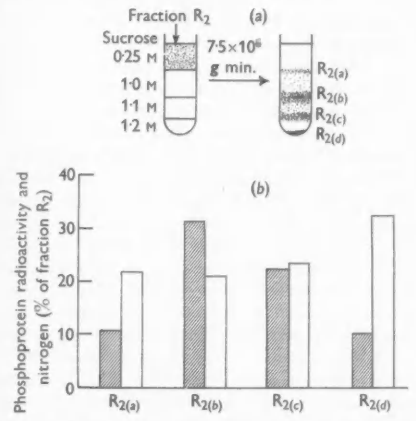

Fig. 3. Refractionation of fraction R_2 (Fig. 2) in a sucrose density gradient. (*a*) Details of the concentrations of sucrose and centrifugal fields used; (*b*) distribution of phosphoprotein radioactivity and nitrogen in the various fractions. ▨, Phosphoprotein; □, nitrogen.

Table 8. *Distribution of phosphoprotein in various sub-fractions from the 'nuclear' fraction of guinea-pig-brain cerebral slices*

Fractionation procedure was as described in Figs. 1–3. Phosphoprotein was determined as radioactive phosphorylserine as described in the Materials and Methods section. Values are the percentage distribution ± S.E.M. of nitrogen and phosphoprotein calculated as a percentage of the quantities taken for each stage in the fractionation.

Stage	Fraction	Nitrogen (% of whole original fraction)	Counts/min. in phosphorylserine (% of whole original fraction)	Counts in phosphorylserine (%) / Nitrogen (%)
I	Nuclei	21·7±1·8 (10)	13·0±2·7 (6)	0·60
	Residue	78·8±2·2 (9)	87·4±3·3 (6)	1·10
	Sum of fractions	100·5	100·4	1·00
II	R_1	17·3±1·5 (4)	11·1±3·5 (3)	0·64
	R_2	45·2±5·1 (4)	42·6±0·6 (3)	0·92
	R_3	23·8±5·4 (4)	9·6±0·6 (3)	0·40
	R_4	23·6±1·4 (4)	6·6±2·4 (3)	0·28
	Sum of fractions	109·9	69·9	0·64
III	$R_2(a)$	23·1±2·6 (3)	11·4±3·4 (3)	0·49
	$R_2(b)$	23·7±3·0 (3)	31·2±1·3 (3)	1·32
	$R_2(c)$	25·8±1·7 (3)	24·8±7·6 (3)	0·96
	$R_2(d)$	36·9±6·7 (3)	11·6±4·5 (3)	0·76
	Sum of fractions	109·5	79·0	—

removal from the animal, was fractionated in a similar sucrose density gradient, 75% of the total succinic dehydrogenase recovered was found in the fraction corresponding to R_3 and 25% in the fraction corresponding to R_2. Hebb & Whittaker (1958) similarly found that when brain mitochondria were fractionated in a sucrose density gradient, 75% of the total succinic dehydrogenase recovered was in particles which passed through a layer of 1·2 M-sucrose. Values for alkaline and acid phosphatase activity in the fractions are also presented in Fig. 4. Whereas fractions R_1–R_4 contained acid phosphatase, only fractions R_2 and R_4 contained alkaline phosphatase. The high alkaline phosphatase activity of R_4 probably reflects the quantities of cell debris and small blood vessels present, which are generally found to contain the highest alkaline phosphatase activity in the brain (Feigin & Wolf, 1955).

DISCUSSION

The work described above has shown that in brain there are at least two phosphoprotein fractions of differing metabolic activities as judged by the incorporation of radioactive phosphorus. The fraction most active *in vivo* is soluble in 0·25 M-sucrose solution and is partly lost to the medium when tissues are incubated under conditions normal for metabolic studies. The second fraction, least active *in vivo*, is associated with particulate material and undergoes little or no change in quantity when slices of brain are incubated in medium. Under these conditions this latter phosphoprotein incorporates radioactive phosphorus to a greater degree than does the phosphoprotein soluble in aqueous sucrose. Furthermore, the extent of the incorporation is markedly increased when the slices, from which it is later derived, are briefly stimulated with electrical pulses. Previous work, which had shown that the phosphorus metabolism of a phosphoprotein of brain is closely connected with the response evoked by applied electrical stimulus, was performed with tissue slices *in vitro*, and so far it has been considered desirable to pay attention to the phosphoproteins exhibiting such properties under these conditions rather than to the others, soluble in 0·25 M-sucrose solution, for which a similar role has not yet been described.

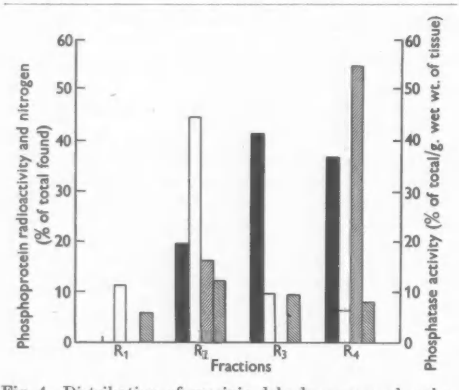

Fig. 4. Distribution of succinic dehydrogenase, phosphoprotein and acid and alkaline phosphatases in subcellular fractions from guinea-pig-brain cortex. ■, Succinic dehydrogenase; □, phosphoprotein; ▨, alkaline phosphatase; ▧, acid phosphatase. Values for acid and alkaline phosphatase in whole cortical dispersions were 2·4 and 11·4 mg. of phenol liberated/g. wet wt. of tissue under the conditions of the test.

The fractionation described above has shown that particles containing a high concentration of the phosphoprotein showing an increased incorporation of ^{32}P in response to electrical pulses can be obtained from slices of brain cortex incubated in suitable media under oxygen. The particles do not appear to be mitochondria, for not only are the mitochondria, when isolated as such both *in vivo* and *in vitro*, extremely poor in phosphoprotein, but in the fractions isolated there is no correlation between the content of succinic dehydrogenase, generally regarded as indicative of the presence of mitochondria, and the phosphoprotein content. Indeed, one fraction which contains phosphoprotein does not contain succinic dehydrogenase. It is well recognized that the mitochondrial fraction from brain is heterogeneous, and it has recently been shown to contain a variety of particles, some of which exhibit little or no succinic dehydrogenase activity (Hebb & Whittaker, 1958). Nevertheless, the fact that preparations of mitochondria containing such particles contain little of the phosphoprotein, together with the evidence of the distribution of succinic dehydrogenase presented above, argues against the particles containing the phosphoprotein arising from this source.

The problems of dealing with the quantitative intracellular localization of a metabolite by means of a property dependent upon its metabolism are considerable, not the least being the possibility of rapid degradation during the fractionation procedure. In this respect the phosphoprotein fraction studied seemed to be reasonably stable, a total loss of 50% occurring during a fractionation occupying some 4–5 hr. This rate is of the order found by Livanova (1957) for the loss of phosphoproteins in liver homogenates at 0°; some 20% was lost in 2–3 hr. and about 30% was lost in 5 hr. There is at present no evidence to suggest that any one group of particles metabolizes the endogenous phosphoprotein more rapidly than another. Indeed the distribution of the acid phosphatase, operative at

a pH near the optimum for tissue phosphoprotein phosphatases (Livanova, 1957; Sundararajan & Sarma, 1957), might suggest the opposite.

In isolation studies of this type, and particularly with brain, where the content of the phospholipids and lipoproteins is high, the possibility that some small particles may be artifacts, produced by dispersion of the tissue, is always present. For a full assessment of this an examination of the particles by different microscopical techniques and comparison with data obtained by examination of whole tissues would be necessary.

SUMMARY

1. A study has been made of the intracellular localization of the phosphoproteins of brain, and in particular of the phosphoproteins whose incorporation of radioactive phosphate is greatly increased when the intact tissue is excited by electrical pulses.

2. When dispersed in isotonic sucrose solution cerebral tissues have been found to contain two major phosphoprotein fractions, one soluble in sucrose solution, the other insoluble. *In vivo*, the incorporation of intracisternally-injected radioactive phosphate was greatest into the soluble phosphoprotein fraction. With slices of brain *in vitro*, incorporation of radioactive phosphate was greatest into the insoluble phosphoprotein fraction.

3. These differences are due to a loss of most of the soluble phosphoprotein from the slice to the incubating medium and to a decreased rate of incorporation of phosphate into the remainder. Incubation in medium had little or no effect upon the total quantity of the insoluble phosphoprotein.

4. After incubation in medium containing radioactive phosphate, tissue slices have been dispersed in isosmotic sucrose and fractionated by differential centrifuging. The bulk of the phosphoprotein was present in the nuclear fraction, the mitochondrial, microsomal and supernatant fractions containing relatively small amounts. Application of electrical pulses to the tissue slices before fractionation showed that the increased incorporation of radioactive phosphate took place largely in the phosphoprotein of the nuclear fraction.

5. The nuclear fraction has been further subfractionated by centrifuging in two sucrose density gradients. The phosphoprotein was concentrated in particles which possess little or no succinic dehydrogenase activity and are not considered to be mitochondria. The acid and alkaline phosphatase activity of the particles has been measured.

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REFERENCES

Agren, G. & Engstrom, L. (1956). *Acta chem. scand.* **10**, 489.
Aldridge, W. N. (1957). *Biochem. J.* **67**, 423.
Brody, T. M. & Bain, J. A. (1952). *J. biol. Chem.* **195**, 685.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 372.
Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*, p. 86. London: Crosby, Lockwood and Son Ltd.
Dawson, R. M. C. & Richter, D. (1950). *Amer. J. Physiol.* **160**, 203.
Engelhardt, V. A. & Lissovskaya, N. P. (1953). *Proc. 19th int. Congr. Physiol., Montreal*, p. 335.
Feigin, I. & Wolf, A. (1955). *J. Neuropath.* **14**, 11.
Harrison, G. A. (1947). *Chemical Methods in Clinical Medicine*, p. 365. London: J. and A. Churchill Ltd.
Heald, P. J. (1956). *Biochem. J.* **63**, 242.
Heald, P. J. (1957). *Biochem. J.* **66**, 659.
Heald, P. J. (1958). *Biochem. J.* **68**, 580.
Hebb, C. O. & Whittaker, V. P. (1958). *J. Physiol.* **142**, 187.
Kamphausen, H. & Morton, R. K. (1956). *Biochem. J.* **63**, 647.
Kratzing, C. C. & Narayanaswami, A. (1953). *Biochem. J.* **54**, 317.
Kuff, E. L. & Schneider, W. C. (1954). *J. biol. Chem.* **206**, 677.
Livanova, N. B. (1957). *Biokhimya*, **22**, 578.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
McIlwain, H. (1951). *Biochem. J.* **49**, 382.
McIlwain, H. & Gore, M. B. R. (1953). *Biochem. J.* **54**, 305.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Neurath, H. (1957). *Advanc. Protein Chem.* **12**, 819.
Rabinowitz, M. & Lipmann, F. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, Sect. 4–68.
Rall, T. W., Sutherland, E. W. & Wosilait, W. D. (1956). *J. biol. Chem.* **218**, 483.
Rossiter, R. J. (1955). *Canad. J. Biochem.* **33**, 477.
Schaffer, N. K., May, S. C. & Summerson, W. H. (1953). *J. biol. Chem.* **202**, 67.
Schneider, W. C. & Potter, V. R. (1943). *J. biol. Chem.* **149**, 217.
Sidbury, J. & Najjar, V. A. (1957). *J. biol. Chem.* **227**, 517.
Strickland, K. P. (1952). *Can. J. Med. Sci.* **30**, 484.
Sundararajan, J. A. & Sarma, P. S. (1957). *Enzymologia*, **18**, 234.
Wosilait, W. (1958). *J. biol. Chem.* **233**, 597.

Comparative Studies of 'Bile Salts'

12. APPLICATION TO A PROBLEM OF RODENT CLASSIFICATION: BILE SALTS OF THE CUTTING-GRASS, *THRYONOMYS SWINDERIANUS**

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The emphasis in these studies so far has been mainly chemical and analytical. With improved analytical methods and growing certainty about the chemical nature and distribution in animal species of at least the C_{24} bile acids, we feel that application of bile-salt comparisons to some biological problems may now be attempted. We report such an application in the following case.

Certain African rodents so strongly resemble animals in somewhat similar habitats in South America that some authorities have supposed them to be closely related. For instance, Simpson (1945) placed the West African cutting-grass, *Thryonomys swinderianus*, and the South American coypu, *Myocastor coypus*, in the same 'superfamily' (Octodontoidea). Wood (1955) discusses rodent classification and concludes that the placing of African forms like *Thryonomys* with the South American species, including the coypu, called by him 'Caviomorpha', is 'morphologically justified, but paleogeographically impossible', in view of the improbability of any geologically recent exchange of mammals between the continents.

The bile salts of the coypu have been carefully examined in this Laboratory (Haslewood, 1954), and we now report a similar investigation of those of the cutting-grass.

RESULTS

It was clearly shown that bile salts of the cutting-grass consisted largely of taurine conjugates. After hydrolysis, chenodeoxycholic (3α:7α-dihydroxycholanic) acid was identified, together with a smaller proportion of deoxycholic (3α:12α-dihydroxycholanic) acid. Ursodeoxycholic (3α:7β-dihydroxycholanic) acid was also detected. The very small ketonic fraction of the bile acids did not appear to contain 3α-hydroxy-7-oxocholanic acid. The bile salts could not be shown to contain any glycine conjugates.

EXPERIMENTAL

General. Melting points are corrected. The Al_2O_3 used was as previously described (Haslewood, 1958). Light petroleum was 'b.p. 40–60°', except where otherwise indicated.

Isolation of bile salts. Gall bladders from 10 specimens of cutting-grass gave by the usual method (Haslewood & Wootton, 1950) 0·76 g. of hygroscopic brown bile salts.

Examination of conjugates. With the system G_3 (for glycine conjugates) and by the methods of paper chromatography described by Haslewood & Sjövall (1954), no free bile acids or glycine conjugates could be detected, although as much as 520 μg. of bile salts was placed at one time on the start-line. With descending chromatography and the system T (for taurine conjugates) of Haslewood & Sjövall (1954), and also the 70% (v/v) acetic acid–water/80% (v/v) 'amyl acetate'–*n*-heptane system of Sjövall (1955), a single spot corresponding to taurodeoxycholate or taurochenodeoxycholate was detected.

Hydrolysis of bile salts and identification of bile acids. Bile salts (40 mg.) of the cutting-grass were heated in a sealed metal bomb with 2·5 N-NaOH (0·2 ml.) and water (0·2 ml.) at 112° for 6 hr. The product was diluted with water and the clear solution treated with 2 N-HCl and NaCl (excess). The semi-solid bile acids were collected, washed with water and dried by evaporation *in vacuo* with ethanol. The residue was left in 2·5% (v/v) H_2SO_4–ethanol (0·5 ml.) for 2·5 days. Water and $NaHCO_3$ (excess) were added and the mixture was extracted three times with ether. The ether was washed with water, dried (Na_2SO_4) and evaporated, leaving 'ethyl esters' (12 mg.).

Ethyl esters (286 mg.) were subjected to Girard separation as previously described (Haslewood, 1954). The 'ketones' (8·2 mg.) could not be identified. The 'non-ketones' (total wt. 272 mg.) on paper chromatography with Bush's (1952) system A gave a spot corresponding to ethyl deoxy- or chenodeoxy-cholate and a faint spot suggesting ethyl ursodeoxycholate or ethyl hyodeoxycholate (3α:6α-dihydroxycholanate).

'Ethyl esters' (12 mg.) in benzene (0·1 ml.) with light petroleum (0·2 ml.) were put on Al_2O_3 (0·5 g.) in a column. Elution was as follows [fraction letter, solvent (vol.), wt. eluted]: *A*, 2:1 (v/v) light petroleum–benzene (4 ml.), 6·4 mg.; *B*, benzene (4 ml.), 0·7 mg.; *C*, ether (10 ml.) 1·8 mg.; *D*, acetone (2 ml.), 0·2 mg.; *E*, ethanol (3 ml.), trace (total recovered, 9·1 mg.). Fractions were each examined by paper chromatography as previously described (Haslewood, 1954) with Bush's (1952) system A.

Only fraction *C* (ether eluate) gave a spot; this corresponded to ethyl deoxy- (or chenodeoxy-)cholate. Dr I. D. P. Wootton compared the infrared spectrum between 900 and 1300 cm.$^{-1}$ of material from fraction *C* with the spectra of mixtures of methyl chenodeoxy- and methyl deoxy-cholate (Wootton & Wiggins, 1953). The spectrum from fraction *C* corresponded to that from a mixture of

* Part 11: Haslewood (1958).

80% (w/w) chenodeoxycholate and 20% (w/w) deoxycholate.

In other experiments, ethyl ester 'non-ketones' were separated on Celite (Johns-Manville Co. Ltd., London, S.E. 11) columns, as described by Anderson, Haslewood & Wootton (1957). The moving phase was light petroleum (b.p. 84–95°) and the stationary phase was 90% (v/v) ethanol–water. The stationary phase (5 ml.) was put on the Celite (10 g.) by shaking it mechanically with the Celite and moving phase (70 ml.) in a stoppered flask for 1 hr. The columns, packed as previously described, were loaded with 'non-ketones' (40–45 mg.). Moving phase (50 ml.) now eluted a fraction (*P*) shown by paper chromatography to contain ethyl deoxy- or chenodeoxy-cholate. A further eluate of moving phase (250 ml.) gave a fraction (*Q*) considerably enriched in the more polar ethyl ursodeoxycholate. In this way, 'non-ketones' (171 mg.) were separated into fraction *P* (127 mg.) and fraction *Q* (44 mg.).

Fraction *P* (40 mg.) was dissolved in acetic acid (0·4 ml.) with acetic anhydride (0·1 ml.). One drop of 8·5 N-$HClO_4$ was added and the dark mixture was left with occasional shaking for 15 min. Water and NaCl (excess) were added and the product was extracted twice with ether. The ether was washed with aqueous NH_3 and water, dried (Na_2SO_4) and evaporated. The residue (43 mg.) crystallized from light petroleum in large colourless prisms (20 mg.), m.p. 103–105°, which after recrystallization from aqueous ethanol had m.p. 106–109°, not depressed by authentic ethyl diacetylchenodeoxycholate (m.p. 106–109°).

Fraction *Q* (44 mg.) was separated by paper chromatography as previously described (Haslewood, 1954) and gave material (4 mg.) which on paper chromatograms with Bush's (1952) system A showed a single spot corresponding to ethyl ursodeoxycholate; this ran distinctly faster than ethyl hyodeoxycholate. This material gave an infrared spectrum (examined through the kindness of Dr R. K. Callow) consistent with the view that it was impure ethyl ursodeoxycholate.

DISCUSSION

Coypu bile has been found to contain cholic (3α:7α:12α-trihydroxycholanic), chenodeoxycholic, ursodeoxycholic and 3α-hydroxy-7-oxocholanic acids, probably all conjugated mainly with glycine (Kazuno & Takuma, 1947; Haslewood, 1954; Haslewood & Sjövall, 1954). It is possible that all these bile acids except cholic and chenodeoxycholic acids are artifacts produced by microbial action in the intestinal tract (Norman & Sjövall, 1958), and this is also true of deoxycholic acid (Lindstedt & Sjövall, 1957) now found in cutting-grass bile. Perhaps, therefore, the only bile acids undoubtedly made in the liver from cholesterol in the two species under discussion are cholic acid and chenodeoxycholic acid; of these, chenodeoxycholic acid is common to both species, and cholic acid, which occurs in coypu bile in small amounts, might be detectable in cutting-grass bile if larger samples were examined.

Ignoring any possible significance (for classification) which the intestinal micro-organisms may have, we may consider the assessment that the 'native' bile salts of both rodent species discussed consist chiefly of chenodeoxycholic acid, with the difference that this is conjugated apparently entirely with taurine in the cutting-grass and largely with glycine in the coypu. This difference we believe to be biologically decisive.

Glycine-conjugated bile salts have been found only in mammals, and their distribution is 'patchy'. A very high proportion of such salts has been observed only in a few vegetarian species (for example, rabbit, coypu, pig). In large collections of coypu- and pig-bile salts from different sources, we have never found more than small proportions of taurine-conjugated compounds. In some other mammals we have not detected any glycine conjugates. Bremer (1956) demonstrated specificity in conjugating with glycine or taurine by liver microsomes from different species (rabbit, rat, chicken); the microsomes *in vitro* gave the same compounds as are known to occur in the bile salts.

It seems therefore unlikely that the kind of conjugation in either species now compared is much affected by diet; the conjugation actually found is almost certainly expressive of inherited liver-enzyme systems.

We think, therefore, that our results support the views of Wood (1955), and other authors quoted by him, that there is no close evolutionary relationship between the coypu and the cutting-grass. In further support of this view, it may be noted that the bile salts of the guinea pig (Imai, 1937), also placed in the Caviomorpha, closely resemble those of the coypu. It may be of interest to make further bile-salt comparisons of members of groups of rodents whose relationships are, morphologically, difficult to determine.

There is, of course, an inescapable element of speculation in any conclusion of this kind made from the nature of a chemical 'character'. A chemical character has the advantage of precision, but its history is unlikely to be revealed by evidence from fossil forms. Deductions made must be inferences from the state of affairs as they exist today; such deductions cannot exclude the possibility of mutations which might invalidate the arguments put forward above.

SUMMARY

1. The bile salts of the West African rodent *Thyronomys swinderianus* (the cutting-grass) have been found to consist chiefly of chenodeoxycholic acid conjugated with taurine. Smaller amounts of (conjugated) deoxycholic and of ursodeoxycholic acids were detected. No glycine conjugates could be found.

2. Comparison of the bile salts with those of the morphologically similar South American coypu (*Myocastor coypus*) previously examined (Haslewood, 1954) supports the view of Wood (1955) that the two rodent species are not closely related in an evolutionary sense.

The authors thank Dr D. C. Erinne for help in the collection of cutting-grass bile. They express their gratitude to Dr R. K. Callow, F.R.S., and to Dr I. D. P. Wootton for their kindness in the determination and interpretation of infrared spectra. The biological problem here discussed was brought to the notice of the senior author by Professor J. E. Webb of the Department of Zoology, University College, Ibadan; the authors thank him and also Dr J. H. Elgood for their interest in this work. They are indebted to Dr G. G. Simpson, of the American Museum of Natural History, New York, for his valuable advice on rodent classification.

REFERENCES

Anderson, I. G., Haslewood, G. A. D. & Wootton, I. D. P. (1957). *Biochem. J.* **67**, 323.
Bremer, J. (1956). *Biochem. J.* **63**, 507.
Bush, I. E. (1952). *Biochem. J.* **50**, 370.
Haslewood, G. A. D. (1954). *Biochem. J.* **56**, 581.
Haslewood, G. A. D. (1958). *Biochem. J.* **70**, 551.
Haslewood, G. A. D. & Sjövall, J. (1954). *Biochem. J.* **57**, 126.
Haslewood, G. A. D. & Wootton, V. (1950). *Biochem. J.* **47**, 584.
Imai, I. (1937). *Hoppe-Seyl. Z.* **248**, 65.
Kazuno, T. & Takuma, T. (1947). *J. Jap. biochem. Soc.* **19**, 14.
Lindstedt, S. & Sjövall, J. (1957). *Acta chem. scand.* **11**, 421.
Norman, A. & Sjövall, J. (1958). *J. biol. Chem.* **233**, 872.
Simpson, G. G. (1945). *Bull. Amer. Mus. nat. Hist.* **85**.
Sjövall, J. (1955). *Ark. Kemi*, **8**, 299.
Wood, A. E. (1955). *J. Mammal.* **36**, 165.
Wootton, I. D. P. & Wiggins, H. S. (1953). *Biochem. J.* **55**, 292.

Studies of Sebum

9. FURTHER STUDIES OF THE COMPOSITION OF THE UNSAPONIFIABLE MATTER OF HUMAN-FOREARM 'SEBUM'*

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The unsaponifiable matter of human-forearm 'sebum' (skin-surface fat) has been shown to contain hydrocarbons (30–46%), cholesterol (14–19%) and wax alcohols (about 20%). The hydrocarbon fraction contains 30–40% of squalene and a normal chain paraffin. A sterol-like substance was detected in the unidentified fraction as were oxidation products of squalene (MacKenna, Wheatley & Wormall, 1950, 1952).

A further examination of the sterol fraction has now been made, and the composition of the wax alcohols has been studied by gas chromatography.

EXPERIMENTAL

Collection of 'sebum'. A bulk collection of sebum was obtained from normal male medical students by immersion of the forearms in acetone (twice-redistilled) as already described (MacKenna *et al.* 1952).

Isolation and fractionation of the unsaponifiable matter. The sebum was saponified as previously described (MacKenna *et al.* 1952) and the unsaponifiable matter isolated. The latter was then fractionated on grade II alumina (Brockman & Schodder, 1941) as described in previous work. In addition, however, the column was washed finally with acetic acid in an attempt to effect a more complete recovery.

Gas chromatography of the wax alcohols. A similar procedure was used to that described for the fatty acids of sebum (James & Wheatley, 1956). The column used had as stationary phase Apiezon M vacuum grease (Shell Chemicals Ltd.) on Celite 545 (Johns-Manville Co. Ltd.) (2·5 parts of Apiezon M to 10 parts of Celite, 40–90 mesh) and was maintained at a temperature of 197° with boiling ethylene glycol. The sample (4·6 mg.) was applied to the top of the column with a micropipette and a flow rate of 60 ml. of N_2/min. was used. Tentative identification of the peaks followed the procedure used for the identification of the fatty acids of sebum (James & Wheatley, 1956); the relative retention times of peak emergence used in these identifications were calculated with reference to octadecanol (cf. James & Martin, 1956). Unsaturated alcohols were removed by the bromination method (James & Martin, 1956) and a second run was made of the saturated members only. Peak areas were measured by the triangulation method and corrected to octadecanol as described by James & Wheatley (1956). From the corrected areas the proportions of each component were calculated.

* Part 8: Boughton, MacKenna, Wheatley & Wormall (1957).

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Table 1. *Chromatographic fractionation on alumina of the unsaponifiable material from a bulked sample of human-forearm sebum*

Fraction	Eluent	Percentage of total unsaponifiable matter	Appearance	Nature*
1	Light petroleum	37·5	Soft white solid	Hydrocarbons
2*a*	5% $CHCl_3$–light petroleum (first 100 ml.)	0·4	Yellow wax	—
2*b*	5% $CHCl_3$–light petroleum	20·6	Yellow wax	Wax alcohols
3*a*	10% $CHCl_3$–light petroleum (first 200 ml.)	3·9	Yellow wax	—
3*b*	10% $CHCl_3$–light petroleum	13·7	White crystalline solid	Cholesterol
4	$CHCl_3$	3·6	Brown–yellow oil	Other sterols
5	Methanol	6·4	Grey wax	—
6	Acetic acid	3·0	Grey–yellow viscous liquid	—
	Total recovered	89·1		

* According to MacKenna, Wheatley & Wormall (1952).

With the column temperatures available, alcohols with chain length longer than 20 carbon atoms could not be detected.

Examination of the sterol fractions

Digitonin precipitation. For a 100 mg. sample the following procedure was used. To a solution of the sterol in 5 ml. of 90% ethanol at 60° an equal volume of a solution of 300 mg. of digitonin in the same solvent was used. The mixture was allowed to stand overnight at room temperature and the white precipitate which formed was filtered and washed with 90% ethanol. The filtrate and washings were evaporated to small bulk, a small amount of digitonin in 90% ethanol at 60° was added and the process repeated. The second crop of digitonide precipitate was added to the first. Sterols not precipitated by digitonin were recovered from the mother liquors and the washings by ether extraction after addition of water.

The cholesterol digitonide was decomposed by dissolving it in 2 ml. of pyridine at 70° and leaving the solution overnight at room temperature (Schoenheimer & Dam, 1933). On adding excess of ether, the digitonin precipitate could be centrifuged off and the cholesterol recovered from the supernatant.

Zimmermann reaction. The method used was that described by Callow, Callow & Emmens (1938).

Girard T' separation of ketonic and non-ketonic sterols. The Girard T complex (Girard & Sandulesco, 1936) was formed by heating an ethanolic solution of the sterol with 0·5 ml. of a 10% solution of the reagent in acetic acid in a boiling-water bath for 30 min. Most of the ethanol and acetic acid was removed under reduced pressure, water was added to the residue and the non-ketonic sterols were extracted with ether. The water-soluble ketonic sterol complex was decomposed by acidifying the aqueous layer with HCl, and the ketonic material was recovered from this by extraction with ether.

Absorption spectra. These were measured with a Unicam SP. 500 spectrophotometer.

RESULTS

Details of the chromatographic fractionation of 1 g. of the unsaponifiable material of human-forearm sebum are given in Table 1. The various fractions were then further examined as described below.

Wax alcohols

The composition of the wax alcohols present in fraction 2*b* (Table 1) was studied by means of gas chromatography. Under the conditions used alcohols of chain length greater than C_{20} could not be detected, but it was calculated that about half the sample consisted of compounds with less than 20 carbon atoms/molecule. The results are given in Table 2 and the gas chromatogram is shown in Fig. 1. The figures for the component fatty acids of forearm sebum (Wheatley, 1957) are given for comparison. The alcohols formed a homologous series similar to the fatty acids of sebum. There were fewer highly branched and unsaturated alcohols in comparison with the acids, and the average chain length of the major alcohol components appeared to be somewhat longer than that of the major acid components. These findings are in general agreement with those of Brown, Young & Nicolaides (1954) who, from mass-spectroscopy evidence, concluded that the average chain length of the alcohols was 20 carbon atoms compared with 16 carbon atoms for the acids.

'*Alkane* 1:2-*diols*'. Fractions 5 and 6 (Table 1) were subjected to periodate oxidation (Karnovsky & Rapson, 1946). Since adequate controls on solvents etc. were run simultaneously it has been concluded that the periodate-oxidizable materials

Table 2. *Quantitative analysis of the wax alcohols and component fatty acids of human-forearm sebum*

Results are expressed as percentage of total material recovered from the gas chromatogram. This is estimated as about 50% of the total sample of alcohols. The analysis of the acids was made by Wheatley (1957). *iso* is used to denote a single methyl branch near the end of the carbon chain.

Type of compound	Alcohol (%)	Acid (%)
n-C_6	0·05	—
n-C_8	0·1	—
iso-C_9	0·1	—
n-C_9	0·1	—
n-C_{10}	0·2	0·3
Highly branched C_{11}	Trace present not estimated	—
n-C_{11}	0·2	0·2
iso-C_{12}	—	0·1
n-C_{12}	0·6	4·4
Highly branched C_{13}	0·2	0·4
iso-C_{13}	—	0·5
n-C_{13}	0·2	0·4
Highly branched C_{14}	—	0·1
iso-C_{14}	0·2	0·3
Mono-unsaturated C_{14}	—	1·9
n-C_{14}	9·8	7·8
Highly branched C_{15}	1·3	1·5
Di-unsaturated C_{15}	—	1·0
iso-C_{15}	—	2·0
Mono-unsaturated C_{15}	—	1·9
n-C_{15}	3·3	4·2
Highly branched C_{16}	—	1·3
iso-C_{16}	0·9	—
Mono-unsaturated C_{16}	—	12·0
n-C_{16}	14·3	22·7
Highly branched C_{17}	0·4	3·4
Di-unsaturated C_{17}	—	1·6
iso-C_{17}	2·8	2·1
Mono-unsaturated C_{17}	3·9	1·4
n-C_{17}	2·4	1·6
Highly branched C_{18}	—	2·9
iso-C_{18}	2·8	—
Mono-unsaturated C_{18}	—	17·2
n-C_{18}	21·4	5·7
iso-C_{19}	1·7	—
Mono-unsaturated C_{19}	1·4	—
n-C_{19}	4·1	—
iso-C_{20}	10·2	—
n-C_{20}	17·5	—

present in these fractions were probably alkane 1:2-diols. It is possible, however, that certain 'oxidation products' of squalene would also react. Calculated as the C_{20} diol the percentages of diols in these fractions were 18·6 and 14·5% respectively.

Sterols

According to MacKenna *et al.* (1952) fraction 3*b* should be composed mainly of cholesterol and fraction 4 of mixed sterols. An attempt was made to fractionate these mixtures further by precipitation of the 3β-ol sterols with digitonin, the residual material being separated into ketonic and non-ketonic portions by means of the Girard T reagent.

Detection of 7-*dehydrocholesterol.* The digitonide from fraction 3*b* was decomposed with pyridine and the recovered cholesterol recrystallized from methanol. The ultraviolet-absorption spectrum of this purified cholesterol fraction was compared with that of a pure specimen of 7-dehydrocholesterol (Fig. 2). Measurement of the peak at 282 mμ, after correction for end-absorption, gave a value of 0·08% of 7-dehydrocholesterol in this purified fraction.

17-*Oxosteroids.* The two keto steroid fractions gave a positive Zimmermann reaction. The time of development and the colour produced were not the same in each case, and were qualitatively different from the colour produced with dehydro*iso*androsterone. There was, however, insufficient material available to investigate this further.

iso*Cholesterol.* Fractions 1, 5 and 6 gave a brownish hue with the Liebermann–Burchardt reagent, whereas fraction 2*b* gave a deep-yellow with a green fluorescence similar to that observed by MacKenna *et al.* (1952). The latter workers ascribed this colour to an unidentified oxidation product of squalene. *iso*Cholesterol, however, under the same conditions gives an orange solution with a green fluorescence, and the absorption spectrum of the solution shows a characteristic absorption maximum at 460 mμ. This absorption peak has been used by Lederer & Tchen (1945) to identify and estimate the presence of *iso*cholesterol in sterol mixtures.

The absorption spectra of the Liebermann–Burchardt colours produced by cholesterol, *iso*-cholesterol and fraction 2*b* are shown in Fig. 3. The curve for fraction 2*b* followed the *iso*cholesterol curve and had a maximum at 460 mμ. After allowing for irrelevant absorption at this wavelength the concentration of *iso*cholesterol was calculated as 0·35% of the fraction. On this basis it appears that some of the chromogen in this fraction, believed by MacKenna *et al.* (1952) to be 'oxidized' squalene, is in fact *iso*cholesterol. The above results have been summarized in Table 3.

DISCUSSION

A further study of the unsaponifiable matter of human-forearm sebum has yielded more information of the complex nature of this lipid. The sterol fraction is obviously a complex mixture with *iso*-cholesterol, 7-dehydrocholesterol and keto steroids occurring in trace quantities.

*iso*Cholesterol, which is a mixture of closely related trimethylsterols, principally lanosterol (Windaus & Tschesche, 1930), is known to be a constituent of the sebum of certain ruminants such as the sheep, llama, goat and dromedary (Lederer & Tchen, 1945), but has not previously been

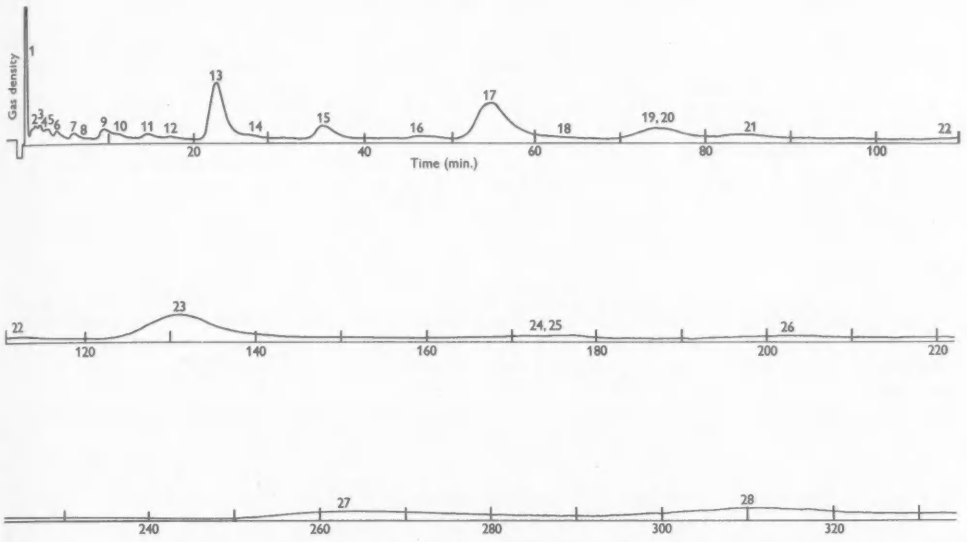

Fig. 1. Analysis of 4·6 mg. of the wax alcohols from human-forearm sebum. Peaks in order of appearance: (1) air introduced in loading column; (2) *n*-hexanol; (3) *n*-octanol; (4) *iso*nonanol; (5) *n*-nonanol; (6) *n*-decanol; (7) highly branched C_{11} alcohol; (8) *n*-undecanol; (9) *n*-dodecanol; (10) highly branched C_{13} alcohol; (11) *n*-tridecanol; (12) *iso*tetradecanol; (13) *n*-tetradecanol; (14) highly branched C_{15} alcohol; (15) *n*-pentadecanol; (16) *iso*hexadecanol; (17) *n*-hexadecanol; (18) highly branched C_{17} alcohol; (19) *iso*heptadecanol overlapping (20) mono-unsaturated C_{17} alcohol; (21) *n*-heptadecanol; (22) *iso*octadecanol; (23) *n*-octadecanol; (24) *iso*nonadecanol overlapping (25) mono-unsaturated C_{19} alcohol; (26) *n*-nonadecanol; (27) *iso*eicosanol; (28) *n*-eicosanol. Column temperature 197°; length 4 ft.; flow rate 60 ml. of N_2/min. Stationary phase: Apiezon M vacuum stopcock grease.

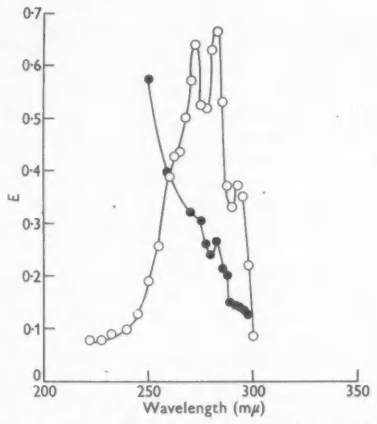

Fig. 2. Ultraviolet-absorption spectra of the ethereal solution of pure 7-dehydrocholesterol (○) and purified cholesterol (●) isolated from human-forearm sebum unsaponifiable matter.

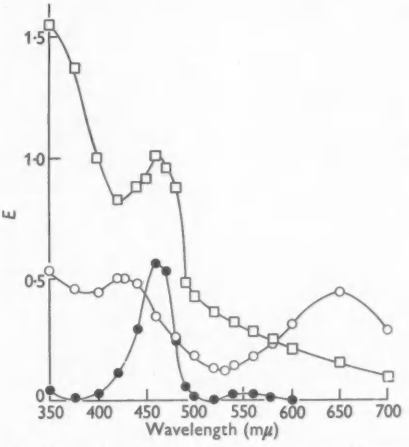

Fig. 3. Absorption spectra of the Liebermann–Burchardt colours produced by cholesterol (○), fraction 2*b* of the unsaponifiable matter of human-forearm sebum (□) and *iso*cholesterol (●).

Table 3. *Some of the constituents of the unsaponifiable matter of human-forearm sebum*

Substance	Detected in fraction no.	Percentage of fraction	Percentage of total unsaponifiable matter	Percentage of whole sebum
7-Dehydrocholesterol	3*b*	0·08	0·003	0·001
*iso*Cholesterol	2*b*	0·35	0·07	0·02
Alkane 1:2-diols	5	18·6	1·62	0·46
	6	14·5		

identified in human sebum. Lanosterol is implicated in the synthesis of cholesterol from squalene (Clayton & Bloch, 1956), and both of these substances occur in human sebum.

7-Dehydrocholesterol is the precursor of vitamin D_3 and is converted into the latter substance by ultraviolet irradiation. It was considered by Helmer & Jensen (1937) that the precursor was secreted in the sebum on to the skin surface and that the vitamin, formed there by irradiation, was reabsorbed through the skin. The precursor has been shown to be present in human (Hentschel & Schindel, 1930) and animal (Koch & Koch, 1941) skin and 7-dehydrocholesterol has been isolated from pig skin (Windaus & Bock, 1937), but previous attempts to detect its presence in the surface-skin lipids (MacKenna *et al.* 1952; Festenstein & Morton, 1952) were unsuccessful. In the present study a trace, only 0·001 %, has been detected in the surface lipids. It has now been shown that the 7-dehydrocholesterol of human skin occurs principally in the epidermis (Wheatley & Reinertson, 1958), the bulk of this must be converted into the vitamin, or otherwise decomposed, before it reaches the skin surface. It now appears more likely that the cells of the Malpighian layer, rather than the sebaceous gland, form the 7-dehydrocholesterol of human skin.

The presence of 17-oxo steroids in human sebum was first claimed by Dubovii (1954) on the basis of the Zimmermann reaction. The presence of ketonic material giving a positive Zimmermann test has been demonstrated in the sterol fraction, but there is insufficient evidence to conclude that this material is, in fact, a 17-oxo steroid.

The study of the wax alcohols of human-forearm sebum by gas chromatography has confirmed and extended the analyses of these compounds already made on hair fats. Brown *et al.* (1954) found odd- as well as even-numbered alcohols, and a monoolefinic series extending from C_{18} to C_{27} as well as the normal saturated alcohols. Hougen (1955) found three homologous series of alcohols present, the normal saturated, the *iso* and the monoolefinic. The analyses described in this paper show yet another group of highly branched compounds, with 11, 13, 15 and 17 carbon atoms, as well as small amounts of the lower members of the normal and *iso* series of alcohols.

The highly branched compounds may be intermediates in the synthesis of squalene from acetate; the C_{15} member may possibly be farnesol. This has been shown to be an intermediate in the biosynthesis of cholesterol (Dituri, Cobey, Warms & Gurin, 1956) and evidence for its presence in human-hair fat has been obtained by Dr N. Nicolaides (unpublished results).

The longer carbon chains of the alcohols compared with the fatty acids, and the presence of relatively fewer highly branched compounds make it unlikely that these two series of compounds have the common biosynthetic route proposed by Rothman & Schaaf (1929).

SUMMARY

1. A further study has been made of the sterol and wax alcohol fractions of the unsaponifiable matter of human-forearm sebum.

2. Gas chromatography was used to study the composition of the alcohols with up to 20 carbon atoms in the molecule. The alcohols formed a homologous series similar to that of the fatty acids of sebum. There were fewer unsaturated and highly branched alcohols in comparison with the acids and the average chain length of the major alcohols appeared to be longer than that of the major acids.

3. The sterol fraction contained traces of 7-dehydrocholesterol (0·003 % of the unsaponifiable matter), *iso*cholesterol (0·07 % of the unsaponifiable matter) and unidentified keto steroids.

4. Alkane 1:2-diols, calculated as C_{20} diols, appear to account for 1·6 % of the unsaponifiable matter.

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REFERENCES

Boughton, B., MacKenna, R. M. B., Wheatley, V. R. & Wormall, A. (1957). *Biochem. J.* **66**, 32.

Brockmann, H. & Schodder, H. (1941). *Ber. dtsch. chem. Ges.* **74**, 71.

Brown, R. A., Young, W. S. & Nicolaides, N. (1954). *Analyt. Chem.* **26**, 1653.

Callow, N. H., Callow, R. K. & Emmens, C. W. (1938). *Biochem. J.* **32**, 1312.
Clayton, R. B. & Bloch, K. (1956). *J. biol. Chem.* **218**, 319.
Dituri, F., Cobey, F. A., Warms, J. V. B. & Gurin, S. (1956). *J. biol. Chem.* **221**, 181.
Dubovii, M. I. (1954). *Ukr. biochem. J.* **26**, 279.
Festenstein, G. N. & Morton, R. A. (1952). *Biochem. J.* **52**, 168.
Girard, A. & Sandulesco, G. (1936). *Helv. chim. acta*, **19**, 1095.
Helmer, A. C. & Jensen, C. H. (1937). *Stud. Inst. Divi Thomae*, **1**, 207.
Hentschel, H. & Schindel, L. (1930). *Klin. Wschr.* **9**, 262.
Hougen, F. W. (1955). *Biochem. J.* **59**, 302.
James, A. T. & Martin, A. J. P. (1956). *Biochem. J.* **63**, 144.
James, A. T. & Wheatley, V. R. (1956). *Biochem. J.* **63**, 269.
Karnovsky, M. L. & Rapson, W. S. (1946). *J. Soc. chem. Ind., Lond.*, **65**, 138.
Koch, E. M. & Koch, F. C. (1941). *Poult. Sci.* **20**, 33.
Lederer, E. & Tchen, P. K. (1945). *Bull. Soc. Chim. biol., Paris*, **27**, 419.
MacKenna, R. M. B., Wheatley, V. R. & Wormall, A. (1950). *J. invest. Derm.* **15**, 33.
MacKenna, R. M. B., Wheatley, V. R. & Wormall, A. (1952). *Biochem. J.* **52**, 161.
Rothman, S. & Schaaf, F. (1929). *Jadassohn's Handb. d. Haut-u. Geschlechtskr.*, **1**, ii, 161. Berlin: Springer Verlag.
Schoenheimer, R. & Dam, H. (1933). *Hoppe-Seyl. Z.* **215**, 59.
Wheatley, V. R. (1957). *J. invest. Derm.* **29**, 445.
Wheatley, V. R. & Reinertson, R. P. (1958). *J. invest. Derm.* **31**, 51.
Windaus, A. & Bock, F. (1937). *Hoppe-Seyl. Z.* **245**, 168.
Windaus, A. & Tschesche, R. (1930). *Hoppe-Seyl. Z.* **190**, 51.

The Inhibition of Bacteriophage Multiplication by Proflavine and its Reversal by certain Polyamines

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Bacteriophages have been known for some time to consist almost entirely of deoxyribonucleic acid and protein, but recently in some *Escherichia coli* phages minor components have been discovered. Hershey (1957) has described two unidentified components, A1 and A2, and a peptide containing aspartic acid, glutamic acid and lysine in trichloroacetic acid extracts of phage T2. Ames, Dubin & Rosenthal (1958) have found the polyamines putrescine and spermidine in phages T2 and T4 and consider them to be identical with components A1 and A2. These findings prompted an examination of a phage in use in this Laboratory known as phage 3, which is similar in many ways to T4. Putrescine and spermidine were found. Before this experiments had been made on the production of incomplete phage particles by the treatment of phage-infected cells with proflavine, as described by De Mars, Luria, Fisher & Levinthal (1953). It was known from the work of Foster (1948) that proflavine could inhibit phage multiplication while permitting cellular lysis. De Mars *et al.* (1953) found that lysis of these cells liberated particles, devoid of nucleic acid, which they believed to be empty phage heads. These they named 'doughnuts' from their appearance in the electron microscope, and suggested that they might be precursors of complete phage. More recently, Kellenberger & Séchaud (1957) have shown that these 'doughnuts' are present in equal amounts in phage lysates whether proflavine is present or not and have cast doubts on the precursor theory. Nevertheless, it seems clear that proflavine can inhibit phage production without interfering with lysis or synthesis of deoxyribonucleic acid (Manson, 1954), although Astrachan & Volkin (1957) maintain that deoxyribonucleic acid from proflavine-treated phage-infected cultures is different from normal phage deoxyribonucleic acid.

The structural similarity between proflavine (2:8-diaminoacridine) and the phage polyamines suggested that the action of the former might be due to interference with the latter. The present paper examines the effect of various polyamines on the inhibition of phage multiplication by proflavine and discusses a possible mode of action of the inhibitor.

MATERIALS AND METHODS

Bacteriophage and host organism. Phage 3 was isolated from Oxford sewage (Fildes & Kay, 1957) and has been found to resemble the well-known coliphage T4 morphologically (Kay & Sampson, 1957) by its possession of the unusual pyrimidine 5-hydroxymethyl cytosine and by its dependence on tryptophan for adsorption. Two mutant forms have been found, one of which ($tryp^+$) requires tryptophan for adsorption to the host bacteria whereas the other ($tryp^-$) adsorbs rapidly in the absence of tryptophan. Phage 3 differs from T4 in numerous details, but in particular it fails to attack the host of T4, *Escherichia coli* B.

A suitable host has been found in another strain of *E. coli*, 518 (3). The phage used in the experiments described in this paper was the tryptophan-dependent ($tryp^+$) mutant. Stocks of this phage were prepared by lysis of *E. coli* 518 (3) grown at 37° in 1 l. batches of medium M5 [composition: KH_2PO_4–Na_2HPO_4 buffer (pH 7·6, 0·033 M-phosphate), NH_4Cl (0·02 M), $MgSO_4$ (0·16 mM), DL-tryptophan (0·1 mM) and glucose, (0·02 M)] in a rotating-flask apparatus of the type described by Mitchell (1949). Sufficient phage was added to give a ratio of two particles per bacterium when the cell density had reached $2·5 \times 10^8$/ml. After 4 hr., by which time lysis had begun, the cultures were placed at room temperature overnight. The lysates were concentrated by evaporation *in vacuo* to one-third of their volume, centrifuged at 1500 *g* to remove bacterial fragments and then at 25 000 *g* to sediment the phage. This was resuspended in KH_2PO_4–Na_2HPO_4 buffer, (pH 7·6, 0·033 M-phosphate) containing 0·015% of serum albumin (human, unheated, not stabilized, kindly supplied by Dr W. d'A. Maycock of the Lister Institute of Preventive Medicine, Elstree, Herts) and again subjected to high- and low-speed centrifuging as before. The resulting pellet of phage is composed of an upper and a lower layer. The upper layer was removed by gentle resuspension in buffer–albumin and discarded, leaving the clear lower layer of phage, which was then resuspended in fresh buffer–albumin and again subjected to low- and high-speed centrifuging. After resuspension in buffer–albumin the phage was examined in the electron microscope and found to consist of undamaged particles almost free of extraneous material. It was then used in the following experiments, after suitable dilution in buffer–albumin. Assays of the phage were made as described by Fildes & Kay (1957). Bacterial cultures of up to 10 ml. were conveniently grown in the T-shaped tubes described by Kay & Fildes (1950) and these were used for the burst-size experiments.

Polyamines and proflavine. Putrescine dihydrochloride, spermine tetrahydrochloride, spermidine phosphate and agmatine sulphate were obtained from Roche Products Ltd. Proflavine was obtained as the hemisulphate from British Drug Houses Ltd. Dilute aqueous solutions of proflavine lost their colour on keeping and a brown pigment was precipitated. They were therefore prepared immediately before use.

Paper chromatography of the polyamines. The propan-2-ol–HCl solvent of Wyatt (1951) separates all four polyamines when applied in 25–30 μg. spots to Whatman no. 1 paper and developed for 66 hr. The positions of the spots are revealed by ninhydrin, after removal of the HCl in the paper by a current of warm air. Agmatine runs the farthest and the other compounds give the following R (agmatine) values: spermine, 0·30; spermidine, 0·51; putrescine, 0·83.

Burst-size experiments. E. coli 518 (3) was grown in M5 medium (10 ml.) containing DL-tryptophan (0·1 mM) and serum albumin (0·02%) in a T tube at 37° until the cell number had reached 10^8 cells/ml. Phage 3 (1 ml.) was then added to give a titre of 8×10^6/ml. and 5 min. allowed for adsorption to take place. The amount of phage left free was determined by centrifuging a 1/10 dilution of the adsorption mixture and assaying the phage in the supernatant. A 1/1000 dilution of the adsorption mixture was made into fresh medium and incubated at 37° in a T tube. Samples were withdrawn at intervals and assayed for phage after suitable dilution. After making a correction for the amount of phage initially unadsorbed a graph was plotted showing the variation in infective centres against time. From this was determined the burst size (the average yield of phage from one infected cell) and the latent period. Under normal conditions phage 3 growing in *E. coli* 518 (3) has a latent period of 26 min. and a burst size of 50–90.

Measurement of inhibition of phage multiplication and its reversal. Burst-size experiments were made as above except that the inhibitor and reversing agents were incorporated into the medium in which the 1/1000 dilution was made. This means that the cells encountered the agent at about the seventh minute. The minimum amount of proflavine required to reduce the burst to zero was found and then burst sizes were determined in the presence of this amount of proflavine and varying amounts of the reversing agents. Controls in normal medium and in medium with proflavine alone were run simultaneously. The degree of reversal is the burst size in the presence of reversing agent and the minimum inhibitory concentration of proflavine expressed as a percentage of the control burst size in the normal medium.

RESULTS

Polyamines in phage 3. If phage 3 (about 10^{12} particles) is applied directly to a paper chromatogram and developed in propan-2-ol–HCl two ill-defined spots are observed corresponding to putrescine and spermidine. The appearance of the spots is greatly improved by using an extract of the phage made in 1·5% (w/v) trichloroacetic acid at room temperature for 15 min. The positions of the two spots coincide exactly with those of putrescine and spermidine. Although this is not conclusive evidence it is thought to be fairly certain that putrescine and spermidine do occur in phage 3, especially as the more exact tests of Ames *et al.* (1958) show the presence of these substances in phage T4. No other ninhydrin-positive spots were found in the phage 3 extracts.

Inhibition of phage multiplication by proflavine. The concentration of proflavine required to inhibit phage multiplication was found by determining burst sizes in the presence of graded amounts of the inhibitor, which was added only to the tubes in which the 1/1000 dilutions were made. This procedure ensures that adsorption and infection take place in normal medium and limits the action of proflavine to the later stages of phage development. The one-step growth curves in the presence of three amounts of proflavine close to the minimum total inhibitory concentration are shown in Fig. 1. Proflavine (μM) reduces the burst size from 80 to 8, 1·5 μM reduces it to 1·5 and 2·0 μM inhibits phage multiplication completely and results in a decline in the infective centres from the end of the latent period onwards. Presumably, in the last case, lysis of the infected cells occurs without the liberation of active phage. In the subsequent experiments on the reversal of phage inhibition proflavine was used at 1·5 μM, as this amount proved to be the minimum

that would regularly reduce the burst size to one or two without danger of over-inhibiting the phage–cell system.

Reversal of proflavine inhibition by polyamines. Putrescine and spermidine were tested at a wide range of levels for ability to restore the power of phage multiplication to infected cells inhibited with proflavine. In each experiment two levels of reversing agent were tested together with one control of normal medium and another with proflavine. More levels than two could not be conveniently tested together in one experiment because it was not possible to take more samples and assay them for phage within the desired time interval of 3 min. The results, which are the product of many individual experiments, each one containing its own normal control from which the degree of reversal is calculated, are given in Table 1. It can be seen that both the polyamines occurring in the phage are capable of reversing proflavine inhibition. Putrescine is most effective and increases in activity with concentration, giving a reversal of 62% at 25·8 mM. Spermidine is not so active (16% at 2·1 mM) and its effect is not increased with increasing concentration. This latter effect will be considered later in this paper.

The reversing effect by the polyamines of phage having been established, it was decided to test other polyamines which do not occur in phage with the expectation that reversal would not take place. Spermidine and agmatine were tried with the results also shown in Table 1. Spermine produced no reversal up to 3·3 mM. Higher concentrations could not be tested because of the precipitation of the insoluble phosphate of spermine. Agmatine, on the other hand, showed considerable reversing power, rising to 87% at 25 mM (the highest level tested). It will be noted that although good reversal can be obtained with both putrescine and agmatine the efficiency of these compounds is very low when referred to the amount of proflavine against which they are tested.

Effect of polyamines on the burst size. In no case has a reversal of 100% been obtained and it was thought that the high concentration of polyamines used might be themselves inhibitory. This was tested by comparison of the burst size given in medium containing varying amounts of the polyamines with that in the normal control. The results are given in Table 2. Agmatine has no effect on the burst size, putrescine is slightly inhibitory at the highest levels tested, spermidine has a marked inhibitory effect and spermine is highly inhibitory. This inhibitory property of spermidine is sufficient to explain its failure to act as a proflavine-reversing agent at the higher concentrations tested.

Attempt to determine whether proflavine is acting as a competitive inhibitor. In order for the inhibitory activity of proflavine with respect to the polyamines to be called competitive it must be shown that the relation between the two is constant

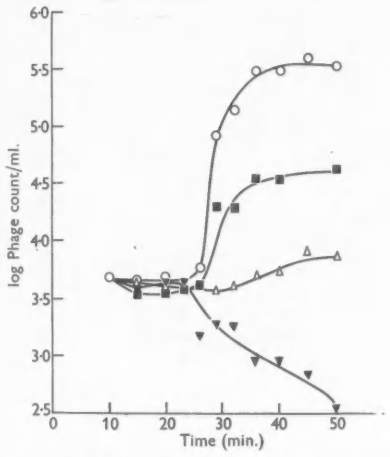

Fig. 1. Effect of proflavine at 1 μM (■), 1·5 μM (△) and 2 μM (▼) on the burst size of phage 3 growing on *Escherichia coli* 518 (3) in M5 medium at 37°. ○, Burst size (80) in medium without proflavine.

Table 1. *Reversal of proflavine-inhibited phage multiplication by polyamines*

The burst size of phage 3 growing on *E. coli* 518 (3) was measured in the presence of 1·5 μM-proflavine with the additions shown, and expressed as a percentage of the burst size found in medium without proflavine.

Polyamine	Concn. (mM)	Percentage reversal of burst size
Putrescine	25·8	62
	12·9	53
	6·8	45
	3·6	26
	1·1	8
	0·36	2
	0·125	0
Spermidine	7·5	7
	2·1	16
	1·0	1·5
	0·42	0
	0·21	0
Agmatine	25·0	87
	17·0	53
	10·0	23
	3·3	3
	1·0	0
	0·33	0
Spermine	10·0 (ppt.)	0
	3·3	0
	1·0	0
	0·33	0

over a range of concentrations. Experiments were set up in which the reversing power of putrescine was tested against two and three times the amount of proflavine found to be the minimum inhibitory amount (1·5 μM). The results are given in Fig. 2. It appears that in the range 1·5–2·6 μM the effect is probably competitive but that when the proflavine concentration is raised further proportionate amounts of putrescine no longer give the same degree of reversal.

Effect of varying the time of addition of proflavine and putrescine to phage-infected cells. In the experiments described so far the infected cells have always encountered the various agents used by dilution into medium containing them in the seventh minute after addition of phage. It was thought that some information about the point of action of the inhibitor and reversing agent might be obtained by varying the time during which these agents were permitted to act. The effect of the delayed addition of proflavine was studied first. The infected cells were diluted 1000-fold into normal medium in a series of tubes to which proflavine (1·5 μM) was added at 7, 10, 15, 20 and 25 min. Burst sizes were determined, with the result shown in Fig. 3. Proflavine at 7 and 10 min. reduced the burst size from 50 in the control to 2. Treatment with proflavine at 15 min. permitted a burst of 4, but at 20 min. a burst size of 30 was obtained. Proflavine at 25 min. did not significantly reduce the burst size below that of the control (48 as opposed to 50). The end of the latent period in the control was at 26 min., whereas that in the culture treated with proflavine at 25 min. was 1–2 min. later.

The effect of delaying the addition of putrescine to cultures inhibited with proflavine was then examined with the purpose of finding whether the latent period was prolonged beyond the normal time. Phage counts were therefore made simultaneously on pairs of cultures at close intervals of time. The results are given in Figs. 4–6. In Fig. 4 the latent periods in a normal culture and one containing proflavine (1·5 μM) and putrescine (12 mM) added together at the seventh minute are compared. It appears that in the reversed culture the latent period is prolonged by about 1 min. In Fig. 5 the latent period in a culture treated with proflavine and putrescine at 7 min. is compared with one treated with proflavine at 7 min. and putrescine at 12 min. There is no apparent difference in the latent periods or the burst sizes. In Fig. 6 the addition of putrescine is delayed until

Table 2. *Effect of polyamines on the burst size of phage* 3 *growing on* Escherichia coli 518(3)

Polyamine	Concn. (mM)	Burst size (percentage of control)
Putrescine	25·0	82
	12·5	94
	6·2	115
	3·1	107
Agmatine	30·0	100
	15·0	100
	7·5	108
Spermidine	9·2	1·3
	4·6	2·5
	2·3	31
Spermine	5·7	0
	1·4	0
	0·14	0
	0·057	75
	0·029	100

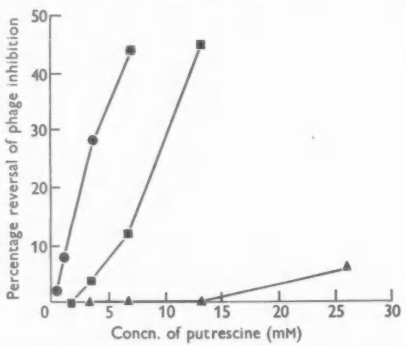

Fig. 2. Reversal of phage multiplication inhibited by proflavine at 1·5 μM (●), 2·6 μM (■) and 3·9 μM (▲) with putrescine.

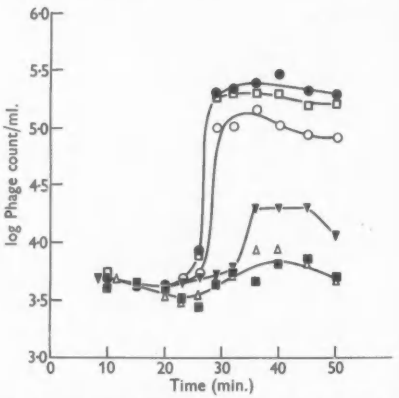

Fig. 3. Inhibition of phage multiplication by proflavine (1·5 μM) added at 7 min. (■), 10 min. (△), 15 min. (▼), 20 min. (○) and 25 min. (□). The control without proflavine (●) gave a burst size of 50.

25 min., in which case there is a prolongation of the latent period by about 6 min. and the burst size is reduced from 29 to 25.

The following conclusions may be drawn from the last four experiments. Proflavine blocks the production of phage when added to an infected culture at 7, 10 or 15 min. When added at 20 min. the blocking is much less complete (60 % of the control burst is produced) and at 25 min. there is no blocking. The treatment of proflavine-blocked cells with putrescine at 7 and 12 min. removes the block and permits the burst to occur with little delay. Putrescine at 25 min., that is, just before the burst would occur in normal cells, reverses the inhibition but only after a delay of about 6 min.

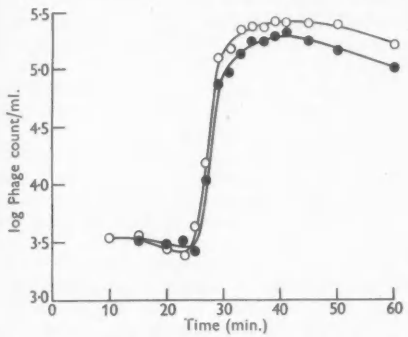

Fig. 4. Comparison between the latent periods and burst sizes of cultures infected with phage 3 in M5 medium (○) and in the same medium with proflavine (1·5 μM) and putrescine (12 mM) at 7 min. (●).

DISCUSSION

Since the discovery of 'doughnuts' or incomplete phage particles by De Mars *et al.* (1953) it has been assumed that proflavine exerts its inhibitory action on phage multiplication at the stage of maturation. This is the point in the developmental cycle at which, it is imagined, the already formed phage nucleic acid is given its coat of protein and converted into an active phage particle by the addition of the complex tail structure. The first of the complete particles appears in the infected cells about halfway through the latent period. Their numbers increase to a maximum at the end of the latent period, when they are dispersed by cellular lysis. The precise site of action of proflavine is not known but from evidence presented in this paper it is suggested it is the putrescine–spermidine–deoxyribonucleic acid complex that is attacked. That a polyamine–deoxyribonucleic acid complex exists in phage is assumed from the results of Ames *et al.* (1958), who found that putrescine enters the host cell together with phage deoxyribonucleic acid on infection and is conserved in the progeny phage. There is sufficient structural similarity between proflavine, itself a polyamine, and putrescine and spermidine to suggest that the inhibitor might be exerting its effect by interference with the polyamines and that the polyamines, if applied in sufficient concentration, would reverse its effect. This suggestion has been confirmed. It was expected that the inhibitor would be competitive but this is so to only a minor extent since, as shown in Fig. 3, a threefold increase in inhibitor cannot be reversed by a similar increase in putrescine, whereas a twofold increase is reversed by a twofold

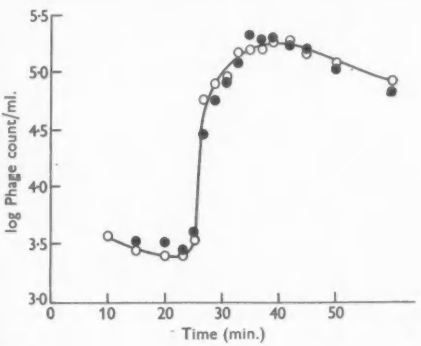

Fig. 5. Comparison between the latent periods and burst sizes of two cultures infected with phage 3 in M5 medium, to both of which proflavine (1·5 μM) was added at 7 min. Putrescine (12 mM) was added to one (○) at 7 min. and to the other (●) at 12 min.

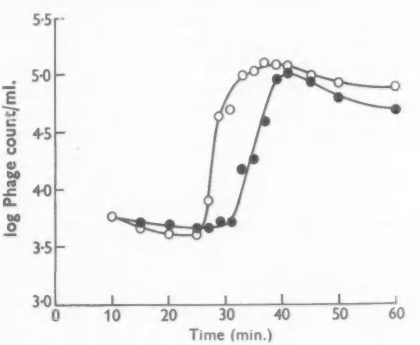

Fig. 6. Comparison between the burst sizes and latent periods of two cultures infected with phage 3 in M5 medium, to both of which proflavine (1·5 μM) was added at 7 min. Putrescine (12 mM) was added to one (○) at 7 min. and to the other (●) at 25 min.

rise in the putrescine concentration. Presumably proflavine at higher concentration exerts other inhibitory effects in which polyamines are not directly concerned.

The function of the putrescine and spermidine in phage may be twofold. They could neutralize the acidic groups in the deoxyribonucleic acid and could by virtue of their two and three functional groups maintain important internucleotide distances. Proflavine is known to form complexes with deoxyribonucleic acid (Peacocke & Skerrett, 1956), and might therefore displace the naturally occurring polyamines. The mutagenic action of proflavine (Brenner, Benzer & Barnett, 1958) might occur by this mechanism. The reversing action of the polyamines could be explained by displacement of the 'foreign' polyamine proflavine and the re-establishment of the normal polyamine–deoxyribonucleic acid relationship. Only when the correct polyamine–deoxyribonucleic acid relationship has been established can the deoxyribonucleic acid be orientated to form the core of the phage head and converted into an active phage particle by the addition of the head membrane and tail structure.

Some information about the time during the latent period at which proflavine acts may be deduced from the experiments shown in Figs. 4–6, in which putrescine is added at intervals to inhibited infected cells. If the times of onset of the bursts are compared it appears that proflavine at 7 min. can be reversed by putrescine at 7 or 12 min. so effectively that the rise in the phage titre occurs within a minute or so of that in the normal control culture. This indicates that the reversal is rapid and that no vital stage in the development of the phage susceptible to proflavine inhibition has been reached by 12 min., since if it had the burst would very likely have been delayed. However, if the putrescine is delayed until 25 min., which is about 1 or 2 min. before the end of the normal latent period, the onset of the burst is delayed by 6 min., although the burst size is not greatly affected. This indicates that the stage at which proflavine acts begins between 12 and 19 min., if it is assumed that 6 min. of 'production time' had been lost by the time the putrescine was added. This period during the development of the phage is the one in which maturation is considered to take place and therefore the hypothesis that proflavine inhibits this stage is supported by the above results. The reversal of the inhibition when putrescine is added, which is assumed to be very rapid, then permits development to completion of phage particles almost equal in number to those produced when putrescine is added early.

SUMMARY

1. Phage 3 contains the polyamines putrescine and spermidine.

2. The multiplication of the phage is inhibited by proflavine and this inhibition is reversed by putrescine, spermidine and agmatine.

3. Spermidine in high concentration inhibits phage multiplication. Spermine is highly inhibitory to phage multiplication but agmatine is inactive and putrescine shows only a slight activity in the highest concentration tested.

4. Proflavine does not damage mature particles but blocks some stage in the intracellular development of the phage.

5. Delayed treatment with putrescine indicates that the block is in the latter half of the latent period, the maturation stage.

6. The proflavine block is thought to be due to interference in the incorporation of the polyamines into the newly synthesized phage deoxyribonucleic acid, which then cannot be orientated to form the core of the phage head.

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REFERENCES

Ames, B. N., Dubin, D. T. & Rosenthal, S. M. (1958). *Science*, **127**, 814.
Astrachan, L. & Volkin, E. (1957). *J. Amer. chem. Soc.* **79**, 130.
Brenner, S., Benzer, S. & Barnett, L. (1958). *Nature, Lond.*, **182**, 983.
De Mars, R. I., Luria, S. E., Fisher, H. & Levinthal, C. (1953). *Ann. Inst. Pasteur*, **84**, 113.
Fildes, P. & Kay, D. (1957). *Brit. J. exp. Path.* **38**, 563.
Foster, R. A. C. (1948). *J. Bact.* **56**, 795.
Hershey, A. D. (1957). *Virology*, **4**, 237.
Kay, D. & Fildes, P. (1950). *Brit. J. exp. Path.* **31**, 338.
Kay, D. & Sampson, J. (1957). *Brit. J. exp. Path.* **38**, 548.
Kellenberger, E. & Séchaud, J. (1957). *Virology*, **3**, 256.
Manson, L. A. (1954). *Fed. Proc.* **13**, 503.
Mitchell, P. (1949). *Nature, Lond.*, **164**, 846.
Peacocke, A. R. & Skerrett, J. N. H. (1956). *Trans. Faraday Soc.* **52**, 261.
Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.

The Differential Effect of Arginine and Canavanine on Growth and Enzyme Formation in *Staphylococcus aureus* 524 SC

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It has been known for many years that bacteria, incubated in the absence of amino acids essential for growth, are incapable of net synthesis of cell protein. Furthermore, Pardee & Prestidge (1956), Yčas & Brawerman (1957) and Gros & Gros (1958) have reported that cultures of *Escherichia coli*, incubated under similar conditions, are unable to synthesize ribonucleic acid or deoxyribonucleic acid. Rogers & Mandelstam (1958), Mandelstam & Rogers (1959) and Hancock & Park (1958) have shown that *Staphylococcus aureus* can synthesize the mucopeptide component of the bacterial cell wall when no protein synthesis is possible through lack of amino acids essential for exponential growth. Prolonged incubation in such media therefore leads to profound changes in cell composition. Little other work, however, has been reported on the effect of low concentrations of essential amino acids on the formation of cell components. Under such conditions, it seemed possible that the synthetic processes dependent on a supply of amino acid might be impaired to different extents. It was of particular interest to see whether the synthesis of nucleic acids and of the various enzyme proteins was affected to the same extent, or whether some processes were more sensitive than others.

The organism chosen for these studies was *S. aureus* strain 524 SC (Rogers, 1953) because it is fully exacting to arginine and is known to synthesize a constitutive extracellular hyaluronidase (Rogers, 1954, 1957), a constitutive extracellular enzyme which can lyse *Micrococcus lysodeikticus* (Richmond, 1959*a*), an intracellular phosphatase and an inducible intracellular β-galactosidase. The hyaluronidase and the lytic enzyme were particularly interesting because they appear in synthetic medium at an increasing differential rate during exponential growth of the organism. It seemed possible that the synthesis of these enzyme proteins might be less closely coupled with the synthesis of other cell proteins and therefore particularly sensitive to a lowered amino acid supply. Further advantages are that cell-wall synthesis in this strain has been studied extensively and protein 'turnover' is very low (about 1%/hr.; Rogers & Mandelstam, 1958; Mandelstam & Rogers, 1959).

The first part of this paper reports experiments carried out to study the formation of various cell components and enzymes when cultures of *S. aureus* 524 were incubated in a fully synthetic medium containing low concentrations of arginine. The second part of the paper describes the effect on the formation of various cell components and enzymes in *S. aureus* 524 of replacing arginine by a structural analogue which could be metabolized by the organisms. L-Canavanine was chosen for this purpose as this amino acid has been reported by a number of workers to compete effectively with arginine (Volcani & Snell, 1948; Horowitz & Srb, 1948; Walker, 1955), although it has not been reported to replace arginine in protein. Previous work on the response of organisms to analogues of essential amino acids has produced no very clear picture. *Escherichia coli* grown in the presence of selenomethionine (a methionine analogue) can synthesize an enzymically active β-galactosidase (Cowie & Cohen, 1957); whereas Gros & Gros (1958) have shown that a phenylalanine-requiring mutant in the presence of *p*-fluorophenylalanine can synthesize a certain amount of nucleic acid but no protein. Brawerman & Yčas (1957) have reported that a tryptophan-requiring mutant of *E. coli* can double in protein and nucleic acid content when grown on azatryptophan (a tryptophan analogue); under these conditions these organisms can synthesize active β-galactosidase (Pardee, Shore & Prestidge, 1956; Pardee & Prestidge, 1958).

MATERIALS

Organisms. Staphylococcus aureus 524 SC (Rogers, 1953; Rogers & Mandelstam, 1958; Mandelstam & Rogers, 1959) was normally maintained in ampoules in the dried state, a new ampoule being opened for each experiment. Dried cultures were opened at intervals to check viability, purity and enzyme production. *Micrococcus lysodeikticus* (NCTC 2665) was used in the preparation of the substrate for lytic-enzyme estimations. The organisms were maintained as described previously (Richmond, 1959*b*).

Media. S. aureus 524 was found to grow well in Hedley–Wright broth containing 2·5% (w/v) of β-glycerophosphate or in casein hydrolysate medium containing 0·5% of glucose, nicotinic acid (1 μg./ml.) and thiamine (1 μg./ml.) (Rogers, 1945).

The fully defined media used were based upon medium 18AA (Table 1). The mixture of amino acids (Roche Biochemicals Ltd.) and nicotinic acid was dissolved in half the final volume of 0·05 M-Na_2HPO_4 and the pH value brought to 6·8 with N-HCl. This solution was autoclaved at 10 lb./in.² for 20 min. The histidine, tryptophan and thiamine (each at ten times the final concentration) were sterilized separately by Seitz filtration. D-Glucose and the 'oligodynamic salt solution' (Pollock & Kramer, 1958) were autoclaved separately. The solution of the amino acid mixture and nicotinic acid was cooled to about 35°, and histidine, tryptophan, glucose and salts were added under sterile conditions together with enough sterile water to bring the medium to the required final volume. Tween 80 (0·04%, v/v) was added to the medium (if required) to minimize surface denaturation of extracellular enzymes (Richmond, 1958). For specific purposes, 18AA medium was modified by omitting arginine [17AA(-Arg) medium]; or arginine and glucose [17AA(-Arg-Glucose) medium].

Amino acids. L-Arginine HCl (Roche Biochemicals Ltd.) was used throughout these studies. I am indebted to Dr J. Mandelstam for a generous gift of L-canavanine, which had been prepared from jack-beans by the method of Kitagawa & Yamada (1932). Ionophoresis of arginine and canavanine in 2% (w/v) $(NH_4)_2CO_3$ buffer, pH 9·0, completely separated the two amino acids, which migrate in opposite directions. No arginine could be detected in a sample of 200 μg. of canavanine thus examined. The amino acids were detected with diacetyl and alkaline α-naphthol by the method of Szafir & Bennett (1953).

Table 1. *Composition of* 18AA *medium* (*see text*)

Amino acid mixture 'A'	Wt. (g.)
DL-Alanine	0·4
L-Aspartic acid	0·4
L-Arginine HCl	0·15
L-Glutamic acid	0·6
Glycine	0·6
DL-*iso*Leucine	0·4
DL-Leucine	0·4
L-Lysine HCl	0·2
DL-Methionine	0·6
DL-Phenylalanine	0·4
L-Proline	0·25
DL-Serine	0·15
DL-Threonine	0·1
L-Tyrosine	0·2
L-Valine	0·4
Nicotinic acid	0·005

Amino acid mixture 'A' was dissolved in 500 ml. of 0·05 M-Na_2HPO_4 and adjusted to pH 6·8 with N-HCl. This was autoclaved at 10 lb./in.² for 20 min., cooled and the following additions were made under sterile conditions:

	Vol. (ml.)
Thiamine HCl (2 mg./ml.)	1·0
L-Tryptophan (2 mg./ml.)	100·0
L-Cystine (2 mg./ml.)	100·0
L-Histidine (2 mg./ml.)	100·0
D-Glucose (4%, w/v)	100·0
'Oligodynamic' solution of salts*	0·1
Sterile water to 1 l.	

* Pollock & Kramer, 1958.

The L-[*carboxy*-^{14}C]leucine was obtained from The Radiochemical Centre, Amersham, Bucks; it was used at a specific activity of 0·05 μc/μmole unless otherwise stated.

Other compounds. I am indebted to Dr H. J. Rogers for a gift of potassium hyaluronate and to Dr J. Mandelstam for *o*-nitrophenyl-β-D-galactoside.

METHODS

Preparation of bacteria for experimental purposes

Dried organisms from ampoules were inoculated into Hedley–Wright broth (12 ml.) containing 2·5% of β-glycerophosphate. This culture was incubated at 35° overnight on a graded shaker (1 foot throw, 86 cyc./min.) (Kantorowicz, 1951). After 16 hr. growth, the culture was diluted into 100 ml. of Hedley–Wright broth and incubation continued until growth was exponential. The organisms were removed from the culture by centrifuging, washed twice with water (15 ml.) and resuspended in a convenient volume of 0·9% NaCl soln. for inoculation. The experimental media were contained in conical flasks of at least five times the culture volume to ensure maximum aeration and were brought to the required temperature and degree of aeration by incubation for 30 min. under experimental conditions before inoculation. Organisms were suspended to an initial culture density of about 150 μg. dry wt. of bacterial cells/ml. and incubated (Rogers, 1954) under conditions described above.

Growth was followed turbidimetrically (Rogers, 1954) and samples of convenient size were removed aseptically as required for analysis or estimation of enzyme activity.

Preparation of samples for enzyme estimations

Samples (containing about 2–3 mg. dry wt. of bacterial cells/ml.) were pipetted into 0·1 vol. of chloramphenicol (100 μg./ml., final concn.). The organisms were centrifuged for 10 min. at 3000 *g*, resuspended in 0·01 M-Na_2HPO_4–KH_2PO_4 buffer, pH 7·0, and assayed for intracellular enzymes (β-galactosidase and phosphatase) and extracellular enzymes (lytic enzyme and hyaluronidase).

Enzyme estimations

β-Galactosidase. A modification of the method of Rickenberg & Lester (1955) was used. Samples of cells (prepared as described above) were incubated for 3 min. at 37° and 1 ml. of *o*-nitrophenyl-β-D-galactoside (1 mg./ml. in 0·01 M-KH_2PO_4–Na_2HPO_4 buffer, pH 7·0) was added. Incubation was continued for 15 min. and the reaction stopped by addition of 1 ml. of M-K_2CO_3 soln. The tubes were transferred to room temperature for 1 hr. to let the colour develop fully, the organisms were removed by centrifuging (3000 *g*, 10 min.) and the concentration of *o*-nitrophenol was estimated by specific absorption at 420 mμ in the Unicam Spectrophotometer, model SP. 500. Enzyme was measured as μmoles of *o*-nitrophenol liberated/mg. dry wt. of bacterial cells/hr. at 37°.

Phosphatase. Phosphatase was estimated by following the release of *o*-nitrophenol from *o*-nitrophenyl phosphate (Sigma Chemical Co., St Louis, Mo., U.S.A.) (1 mg./ml. in 0·01 M-phosphate buffer, pH 7·0) under exactly the same conditions as were used for estimation of β-galactosidase. Enzyme activity was expressed in the same units.

Lytic enzyme. Lytic enzyme was estimated by its ability to solubilize cell suspensions of *Micrococcus lysodeikticus* (Richmond, 1959*a*). Details of the estimation together with method of preparation of the substrate and definition of the enzyme unit were exactly as described for estimation of the *Bacillus subtilis* R lytic enzyme (Richmond, 1959*b*).

Hyaluronidase. Preparations of hyaluronidase were made by growing *Staphylococcus aureus* 524 in casein hydrolysate media under the conditions described above until the culture density reached about 1·0–1·2 mg. dry wt. of bacteria/ml. The culture medium containing the enzyme was cleared of organisms by centrifuging, dialysed for 24 hr. against 100 vol. of 0·001 M-KH_2PO_4–Na_2HPO_4 buffer, pH 7·0, and concentrated by freeze-drying. Estimation of this enzyme was carried out as described by Rogers (1954) and expressed as turbidity reducing units/ml. (TRU/ml.).

Preparation of samples for chemical fractionation

Samples (containing approx. 1 mg. dry wt. of organisms) were removed from the culture, pipetted into an equal volume of 10% (w/v) trichloroacetic acid at 4° and stored at that temperature for 1 hr. The precipitate was removed by centrifuging and fractionated by a modification of the procedures of Ogur & Rosen (1950) and McQuillen & Roberts (1954).

(1) *Preparation of lipid and 'alcohol-soluble' protein fractions.* The precipitated material was washed with 5% (w/v) trichloroacetic acid (3 ml.) at 4° and then extracted twice at 75° for 30 min. with 2 ml. of ethanol–water (3:1, v/v). The extracts were removed on the centrifuge, combined and split into an ether-soluble (lipid) and water-soluble (alcohol-soluble protein) fraction by addition of 2 vol. of water and 2 vol. of ether. The two layers obtained in this way were separated and taken to dryness *in vacuo*.

2*a*. *Simultaneous extraction of ribonucleic acid and deoxyribonucleic acid.* The insoluble residue from (1) was suspended in 5% trichloroacetic acid (3 ml.) and heated on a water bath at 95° for 30 min. The supernatant was removed on the centrifuge, and the extraction with trichloroacetic acid and centrifuging were repeated. The acid was removed from the combined extracts by shaking five times with an equal volume of ether. The ether layer was discarded and the aqueous layer taken to dryness at 100° in a stream of air.

2*b*. *Separate extraction of ribonucleic acid and deoxyribonucleic acid.* Ribonucleic acid (RNA) was extracted from the residue from (1) by treatment with *N*-perchloric acid overnight at 2° (Ogur & Rosen, 1950). The perchloric acid (containing the RNA) was removed by centrifuging. Deoxyribonucleic acid (DNA) was estimated directly on the residue.

3. *Preparation of 'protein + cell-wall fraction'.* The protein + cell-wall fraction was prepared by washing the insoluble residue from (1) + (2*a*) successively with aqueous ethanol and ether. The washings were discarded.

Estimation of chemical components

Nucleic acids. RNA was estimated by its specific absorption at 260 mμ. A 1 mg./ml. solution of pure RNA was assumed to have a specific absorption of 28 extinction units (Gale & Folkes, 1953). DNA was estimated as deoxyribose by the method of Burton (1955).

Bacterial cell wall. Cell wall was estimated by the concentration of material reacting as amino sugar in hydrolysates of the protein + cell-wall fraction. About 1·5 mg. of protein + cell wall was hydrolysed (4 N-HCl; 4 hr., 105°) in a sealed tube. This process liberates free glucosamine and muramic acid with negligible destruction (Perkins & Rogers, 1959). The HCl was removed at 100° in a stream of air, residual traces of acid being removed by successive addition of several volumes of water and evaporation to dryness. The residue was dissolved in a known volume of water and the concentration of amino sugar present estimated by the method of Elson & Morgan (1933).

Radioactivity determination. Material to be estimated for radioactivity was taken to dryness at 105° (if necessary) and dissolved or suspended as finely as possible in aqueous 75% (v/v) acetone (1 ml.). Samples (0·3 ml.), containing less than 0·5 mg. dry wt. of material, were transferred to stainless-steel planchets (1 cm.²), dried at 50° and radioactivity was measured by use of an end-window Geiger–Müller tube and scaler. Samples were counted until at least 10^3 counts above background had been registered and it was assumed that the samples counted here were infinitely thin. Background radioactivity was measured before and after counting; 5·0 μmc of L-[*carboxy*-^{14}C]leucine gave an average of 614 counts/min. in the apparatus used.

Terminology

Bacterial growth rate is defined as the increase in bacterial cell dry wt./unit time: $\Delta G/\Delta T$. Differential rate of enzyme formation is defined as the increase in enzyme activity in a given time/increase in bacterial dry wt. in the same period: $\Delta E/\Delta G$ (Monod, Pappenheimer & Cohen-Bazire, 1952).

RESULTS

Growth and enzyme formation in a fully defined medium

Staphylococcus aureus 524 grew well in the fully synthetic 18AA medium under the conditions described above (see Methods section). The generation time of the culture in this medium was about 50–55 min.; organisms cultured in casein hydrolysate medium or Hedley–Wright broth (Rogers, 1945) have a generation time of 40 and 35 min. respectively. In 18AA medium, growth continued to a final density of about 3·5–4·0 mg. dry wt. of bacterial cells/ml., although at densities greater than about 1·4 mg./ml. growth ceased to be truly exponential; this should not affect the experiments described in this paper, which were normally carried out over a range in bacterial-cell density from 100 to 800 μg./ml. During exponential growth over this range of cell densities, the rate of total protein, RNA, DNA and cell-wall formation remained constant.

Phosphatase. No phosphatase activity was released into the growth medium during exponential growth in 18AA medium and the differential rate of formation of cell-bound enzyme under these conditions remained constant, provided that the concentration of inorganic phosphate in the medium did not change markedly. Concentrations

of phosphate greater than 0·04 M caused suppression of enzyme formation and no enzyme was detectable in phosphate concentrations greater than 0·15 M.

Lytic enzyme and hyaluronidase. Both these enzymes are liberated into the growth medium during exponential growth in Hedley–Wright broth, casein hydrolysate medium or 18AA medium (Rogers, 1954, 1957; Richmond, 1959*a*). At any instant, the extracellular enzyme activity of either enzyme represented more than 80% of the total activity present in the culture after disruption in the Mickle disintegrator. No hyaluronidase activity was detected (by methods used in these studies) at culture densities less than about 150 μg. dry wt. of bacterial cells/ml., and no lytic enzyme below about 70 μg./ml. Subsequently, the differential rate of formation of both enzymes increased rapidly for the duration of exponential growth (Richmond, 1959*a*).

β-Galactosidase. Galactose (optimum concentration: 2%, w/v) was found to be the best inducer of β-galactosidase formation; lactose induced poorly (1–2% of the effect of galactose at equivalent molar concentrations) and thiomethyl-β-D-galactoside (Hogness, Cohn & Monod, 1955; Creaser, 1955; Monod, 1956) was inactive. The induction by galactose was completely suppressed by the presence of glucose. When organisms were incubated at very high suspension densities (about 1·5 mg. dry wt. of bacterial cells/ml.) in media containing mixtures of glucose and galactose, induction was delayed by an amount proportional to the initial glucose concentration; presumably induction was suppressed while any glucose remained in the culture medium. Cultures grown in a mixture of the two sugars showed a typical 'diauxie' growth curve (Monod, 1942). Attempts to liberate the enzyme from the organisms by treatment with toluene (Monod, Cohen-Bazire & Cohn, 1951) or cetyltrimethylammonium bromide (Salton, 1951), by disruption in the ultrasonic disintegrator (Gale & Folkes, 1955) or the Mickle disintegrator (Mickle, 1948), or in the press designed by Hughes (1951), resulted in complete loss of activity (cf. Creaser, 1955).

Growth and changes in enzyme components on incubation in the absence of arginine

Exponential growth was not possible when arginine was omitted from the growth medium. Fig. 1 shows that culture inoculated to an initial density of about 200 μg. dry wt. of bacterial cells/ml. and incubated in 17AA(-Arg) medium, increased in turbidity about 85% in the course of incubation for 3 hr., after which there was no further change. Changes occurring in composition of *S. aureus* 524 during incubation under these conditions were studied as follows.

Formation of ribonucleic acid and deoxyribonucleic acid. There was a net increase of about 13% in the RNA content of cultures grown in the absence of arginine; there was no significant increase in the content of DNA in the same experiment. Measurement of the accumulation of material with a specific absorption at 260 mμ, (*a*) in the cold 5% trichloroacetic acid-soluble 'pool' or (*b*) in the culture medium, showed that these results could not be due to synthesis at the control rate accompanied by the rapid breakdown and release of RNA from the organisms. The rate of RNA synthesis was about half that reported by Mandelstam & Rogers (1959) for organisms of the same strain incubated in a buffered salts medium containing glucose and glycine.

Incorporation of L-[carboxy-^{14}C]*leucine into the protein and lipid. S. aureus* 524 was found to incorporate radioactivity from [*carboxy*-^{14}C]leucine predominantly into the lipid, protein + cell-wall fraction and alcohol-soluble protein fraction of the cells. Organisms were inoculated into 18AA medium containing mM-[*carboxy*-^{14}C]leucine to an initial density of about 200 μg. dry wt. of organisms/ml. and incubated for 1 hr. Growth and incorporation were stopped by the addition of an equal

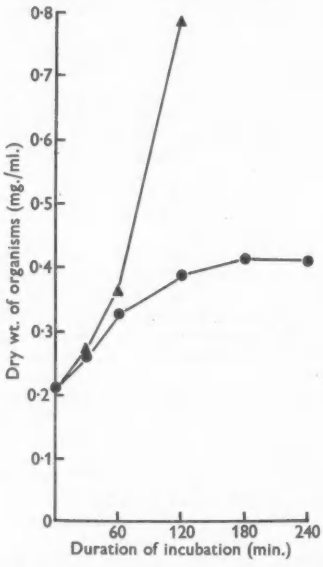

Fig. 1. Growth of organisms in fully synthetic amino acid media. ▲, 18AA medium; ●, 17AA(-Arg) medium. Initial culture density: 215 μg. dry wt. of organisms/ml.

volume of cold 10% trichloroacetic acid containing 1% (w/v) of L-[^{12}C]leucine and the resulting precipitate was fractionated to produce lipid, alcohol-soluble protein, RNA, DNA and protein + cell-wall fractions as described above. In such an experiment, 41% of the radioactivity appeared in the lipid, 45% in the protein and 4·3% in the alcohol-soluble protein. As the cell wall of *S. aureus* 524 contains less than 2% (by wt.) of leucine (J. Mandelstam & H. R. Perkins, personal communication) the incorporation of radioactivity into the protein + cell-wall fraction is a valid measure of protein synthesis provided that the fraction is free from contaminating lipid. Fig. 2 shows the time course of incorporation of [^{14}C]leucine in a similar experiment.

When organisms were incubated in 17AA(-Arg) medium in the presence of [*carboxy*-^{14}C]leucine the rate of incorporation into lipid was initially about 60% of that in the control culture in 18AA medium, but fell to about 35% in the course of incubation for 60 min. On the other hand, practically no incorporation of radioactivity into the protein + cell wall occurred during incubation for 150 min. in the 17AA medium. The incorporation of radioactivity into the alcohol-soluble protein fraction continued at a reduced rate under these conditions; accurate measurements were not possible because of the low levels of incorporation and the high inherent risk of contamination with lipid.

Cell-wall synthesis. The mucopeptide component of *S. aureus* 524 cell wall contains the amino acids lysine, glycine, alanine and glutamic acid together with the amino sugar derivatives *N*-acetylglucosamine and *N*-acetylmuramic acid (Mandelstam & Rogers, 1959; Perkins & Rogers, 1959). As most of the mucopeptide component of the wall is insoluble in hot 5% trichloroacetic acid (Hancock & Park, 1958), mucopeptide synthesis was measured by following the amount of amino sugar liberated from the protein + cell-wall fraction on hydrolysis (see Methods section). Fig. 3 shows that the quantity of trichloroacetic acid-precipitable amino sugar increased about 85% by weight in the course of incubation for 3 hr. This corresponds to an increase in cell-wall content of about 170 μg./mg. initial dry wt. of organisms. Mandelstam & Rogers (1959) have reported that omission of glucose from their incubation mixtures resulted in loss of ability to synthesize cell wall. Similarly, in these experiments, omission of glucose from 17AA(-Arg) medium prevented increase in turbidity and synthesis of the mucopeptide component of the cell wall.

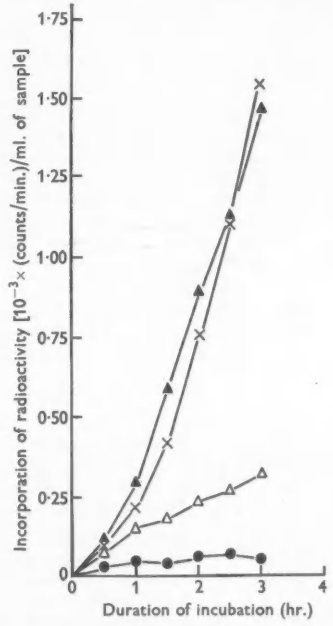

Fig. 2. Time course of incorporation of radioactivity from [*carboxy*-^{14}C]leucine into the lipid fraction (×, △) and the protein + cell-wall fraction (▲, ●) of organisms incubated in media with and without arginine. Initial suspension density: 210 μg. dry wt. of organisms/ml. [^{14}C]Leucine concentration: mM at 0·05 μc/μmole. Media: 18AA (×, ▲); 17AA(-Arg) (△, ●).

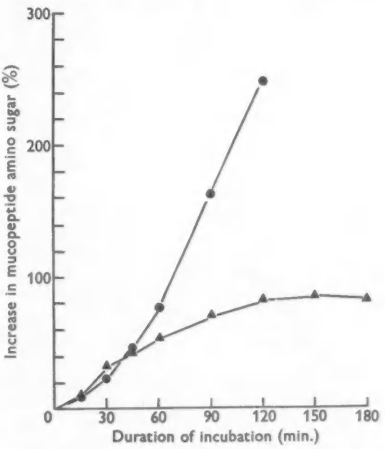

Fig. 3. Formation of 'mucopeptide' amino sugar during incubation of organisms in 18AA medium (●), and in 17AA(-Arg) medium (▲). Initial culture density: 200 μg. dry wt. of organisms/ml. Mucopeptide amino sugar was estimated as described in the Methods section.

Enzyme formation. No formation of hyaluronidase, lytic enzyme or phosphatase could be detected during incubation of cultures in 17AA(-Arg) medium. Furthermore, it was not possible to induce cultures to form β-galactosidase although this enzyme was formed abundantly in control cultures after induction.

Growth in limited concentrations of arginine

The effect of low concentrations of arginine was studied by augmenting the basal 17AA(-Arg) medium with small quantities of arginine. Organisms were inoculated into batches of 17AA(-Arg) medium to which were added 2, 4, 5, 10 and 100 μg. of arginine/ml.; a further culture incubated in the absence of arginine acted as control. Fig 4 shows that the initial growth rate was the same in the various concentrations of added arginine but that the total rapid growth obtained depended (up to about 5 μg./ml.) on the amount of this amino acid added. The total growth obtained on 2, 4 and 5 μg./ml. indicated that 1 μg. of arginine supported the formation of about 60 μg. of cell material. Addition of higher concentrations of arginine did not lead to increased total growth, presumably because some other factor became limiting before the arginine was exhausted. At low concentrations, the rapid phase of growth was followed by a period of slow increase in turbidity. Changes in cell components during this period were essentially similar to those occurring in the absence of added arginine.

Nucleic acid synthesis. The formation of RNA and DNA was studied in cultures incubated in basal medium 17AA(-Arg) containing 2, 4 and 100 μg. of arginine/ml. A net increase in RNA and DNA continued throughout the period of rapid growth and there was no change in the differential rates of synthesis of either nucleic acid at low amino acid concentrations.

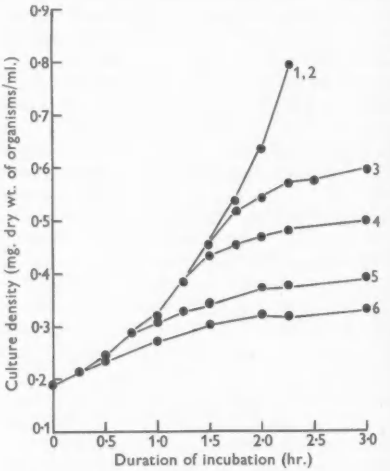

Fig. 4. Growth curves obtained by incubating organisms in the basal 17AA(-Arg) medium containing various added amounts of arginine. Arginine concentrations (μg./ml.): 1, 100; 2, 10; 3, 5; 4, 4; 5, 2; 6, nil. Initial culture density: 180 μg. dry wt. of organisms/ml.

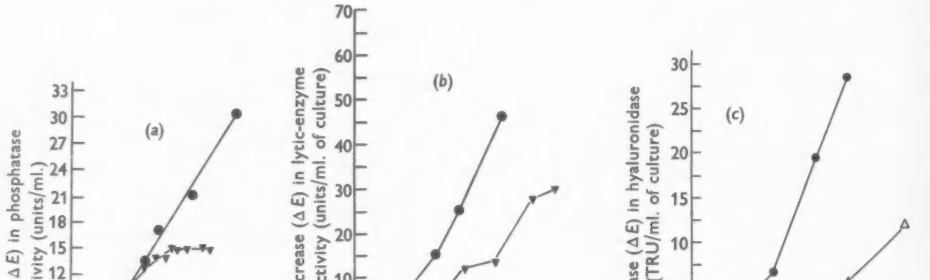
(a)
Increase (Δ E) in phosphatase activity (units/ml.)
Increase (Δ G) in culture density (mg. dry wt. of organisms/ml.)
(b)
Increase (Δ E) in lytic-enzyme activity (units/ml. of culture)
A
B
C
Increase (Δ G) in culture density (mg. dry wt. of organisms/ml.)
(c)
Increase (Δ E) in hyaluronidase (TRU/ml. of culture)
A
B
Increase (Δ G) in culture density (mg. dry wt. of organisms/ml.)

Fig. 5. Differential plots ($\Delta E/\Delta G$) of phosphatase (*a*), lytic enzyme (*b*) and hyaluronidase (*c*) formation in cultures grown on basal 17AA(-Arg) medium supplemented with arginine (μg./ml.); (●), 100; (▼), 4; (▲), 2; (△), 10 (*c* only); (■), nil. Initial culture density: 205 μg. dry wt. of organisms/ml. Points *A*, *B* and *C* (*b*), *A* and *B* (*c*) are referred to in the text.

Phosphatase. Variations in arginine concentration had no significant effect on the differential rate of phosphatase synthesis (Fig. 5*a*). The total amount of enzyme formed in the presence of 4 μg. of arginine/ml. was almost exactly twice that formed in 2 μg./ml.; higher concentrations did not lead to a proportional increase in the amount of enzyme formed.

Lytic enzyme and hyaluronidase. The formation of both these enzymes was impaired at low concentrations of arginine. There was a lag in all cultures before lytic enzyme (Fig. 5*b*) appeared. In 100 μg. of arginine/ml., lytic enzyme was detectable after incubation for about 15 min. (*A*, Fig. 5*b*) and thereafter it accumulated at an increasing differential rate for at least 60 min. In the presence of 4 μg./ml., however, although growth was as rapid as in 100 μg./ml., enzyme formation did not commence for about 40 min. (*B*, Fig. 5*b*) and then occurred at a lower differential rate. In 2 μg./ml. the lag period was about 90 min. (*C*, Fig. 5*b*) and the differential rate about one-quarter of that in 4 μg./ml. Parallel experiments confirmed that, under these conditions, the extracellular enzyme activity was closely related to the total activity detectable after disruption of the organisms in the Mickle disintegrator. Fig. 5*c* shows the appearance of hyaluronidase in the same experiment. After a lag of about 20 min. (*A*, Fig. 5*c*) enzyme formation occurred in the culture containing 100 μg. of arginine/ml.; a similar culture incubated in 10 μg. of arginine/ml. showed some enzyme formation after incubation for 85 min. (*B*, Fig. 5*c*). No enzyme was detected at any time in the other cultures. To check that these results were not due to enzyme inactivation under these conditions, crude *S. aureus* 524 hyaluronidase was added to each culture at the beginning of a similar experiment (final concentration: 30 TRU/ml.; prepared as described in the Methods section). After incubation for 150 min. (Table 2) enzyme activity in the culture containing 100 μg. of arginine/ml. increased about twofold; that in 4 and 2 μg./ml. and in the control culture remained approximately constant.

β-Galactosidase. The differential rate of β-galactosidase synthesis was studied in a similar way to that of phosphatase except that 17AA(-Arg-Glucose) medium plus galactose was used in place of 17AA(-Arg) medium. All the cultures incubated in the presence of arginine underwent a lag before growth and enzyme formation occurred; the duration of the lag was slightly increased in lower arginine concentrations. Subsequently, the rate of enzyme production increased either until halted by lack of arginine or until a maximum rate was achieved after about 90 min. incubation. A differential plot of β-galactosidase formation (Fig. 6) shows that, even in the presence of 100 μg. of

Table 2. *Stability of hyaluronidase under various conditions of incubation*

Cultures were incubated in the 17AA(-Arg) medium with addition of arginine and crude staphylococcal hyaluronidase, as shown.

Additions		Hyaluronidase activity (TRU/ml.) after incubation	
Arginine (μg./ml.)	Hyaluronidase (TRU/ml.)	30 min.	150 min.
100	30	38	64
4·0	30	28	31
2·0	30	28	32
Nil	30	27	28

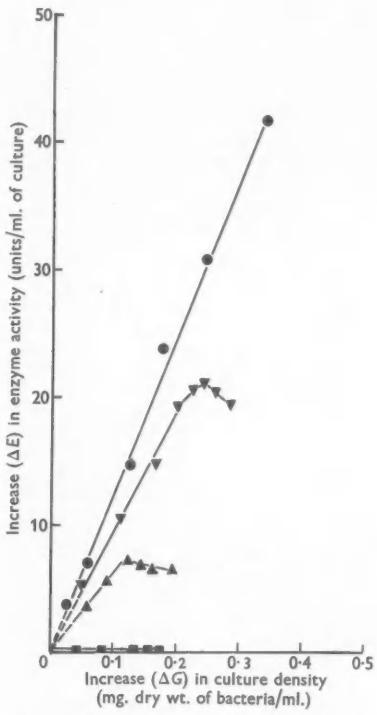

Fig. 6. Differential plot ($\Delta E/\Delta G$) of β-galactosidase formation in cultures incubated in media containing various concentrations of arginine. Media: basal 17AA(-Arg-Glucose) medium containing 2% of galactose and arginine (μg./ml.) as follows: (●), 100; (▼), 4; (▲), 2; (■), nil. The mean generation time of organisms grown in 17AA(-Arg-Glucose) augmented with arginine and galactose was approx. the same as that achieved in 18AA medium.

arginine/ml., the differential rate was approximately linear. Possible reasons for this are discussed below (see Discussion). Fig. 6 also shows that the differential rate of β-galactosidase formation varied with the concentration of the arginine in the medium. In 4 and 2 μg. of arginine/ml. the differential rates are about 70 and 50 % of the rate achieved in 100 μg./ml.

To see whether the cessation of β-galactosidase formation at low concentrations of arginine was due to limitation by the amino acid, a further 2 μg./ml. was added to a culture which had been incubated in 2 μg./ml. and had ceased to grow and to form enzyme. Growth and enzyme formation recommenced immediately and continued until the second batch of arginine was exhausted; addition of more arginine elicited a further burst of growth and enzyme formation. The differential rate of enzyme formation increased with successive batches of arginine until it reached the rate characteristic of organisms grown in 100 μg./ml.

Effect of replacement of L-*arginine by* L-*canavanine*

The effect of canavanine was studied on organisms which had been grown in 17AA(-Arg) medium containing 2 μg. of arginine/ml. A culture growing exponentially in Hedley–Wright broth was centrifuged, washed twice with 17AA(-Arg) medium and resuspended in the basal 17AA(-Arg) medium to which was added 2 μg. of arginine/ml. Incubation was continued until rapid growth ceased (phase 1), and the culture was then divided into four parts (Fig. 7) to which additions were made as follows: phase II, culture 1, nil; culture 2, 2 μg. of arginine/ml.; culture 3, 100 μg. of arginine/ml.; culture 4, 50 μg. of canavanine/ml. Fig. 7 shows that growth occurred immediately in cultures 2 and 3, and that growth in culture 2 slowed markedly after about 80 min. when the supply of arginine was exhausted. Culture 4, on the other hand, increased in turbidity slowly for the first 40 min. but thereafter more rapidly, reaching a maximum rate after about 75 min. Growth slowed after about 150 min., at which point the culture had increased in turbidity about 80 %. Under the conditions of this experiment, canavanine can be detected in the protein + cell-wall fraction of the organisms (M. H. Richmond, unpublished results) and the effect of this phenomenon on enzyme formation and changes in cell composition was studied as follows.

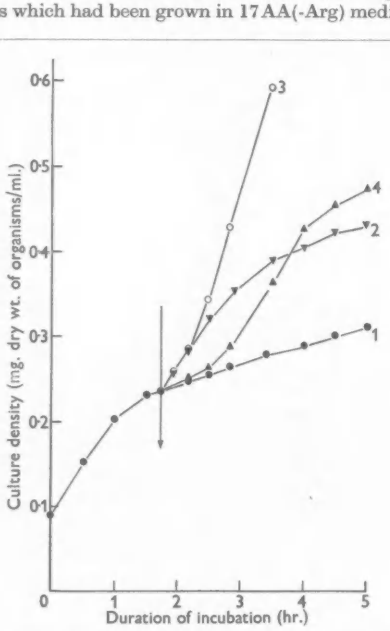

Fig. 7. Effect of arginine and canavanine on the growth of *S. aureus* 524. Media: phase I; basal 17AA(-Arg) medium + 2 μg. of arginine/ml. Culture was divided at the arrow into four parts and additions were made as follows: 1 (●), nil; 2 (▼), 2 μg. of arginine/ml.; 3 (○), 100 μg. of arginine/ml.; 4 (▲), 50 μg. of canavanine/ml.

Nucleic acid synthesis. Organisms of this strain can synthesize RNA and DNA in media containing 1–2 μg. of arginine/ml. When the arginine in such an experiment was replaced by 50 μg. of canavanine/ml., no significant increases in RNA and DNA were observed over the control values obtained in the absence of either amino acid.

Cell-wall synthesis. The increase in trichloroacetic acid-precipitable amino sugar was studied in organisms grown in the basal 17AA(-Arg) medium (culture 1), in similar medium augmented with 2 μg. of arginine/ml. (culture 2), with 100 μg. of arginine/ml. (culture 3) and with 50 μg. of canavanine/ml. (culture 4). The progress curves (Fig. 8) show that organisms incubated in the presence of canavanine were capable of forming cell-wall material at about the same rate as cultures incubated in 2 μg. of arginine/ml. The total quantities of wall synthesized in the presence of 2 μg. of arginine/ml. or 50 μg. of canavanine/ml. are about 100 and 140 μg./mg. initial dry wt. of organisms respectively. Cell-wall preparations of *S. aureus* 524 contain less than 1 % (by wt.) of arginine (H. R. Perkins & J. Mandelstam, personal communication) and attempts to demonstrate the incorporation of canavanine into cell-wall preparations prepared by the method of Hancock & Park (1958) were unsuccessful.

Enzyme formation and protein synthesis: phosphatase, hyaluronidase and lytic enzyme. No increase in the level of phosphatase activity, or appearance of hyaluronidase or lytic enzyme, could be detected in cultures incubated for 3 hr. in the basal

17AA(-Arg) medium containing 50 μg. of canavanine/ml. Addition of 50 μg. of canavanine/ml. to cultures growing in the basal 17AA(-Arg) medium containing 2, 10 or 100 μg. of arginine/ml. had no effect on the differential rates of enzyme formation regardless of the time of addition of the analogue.

β-Galactosidase. The effect of canavanine on β-galactosidase synthesis was studied in cultures partly induced by a period of growth (phase I, Fig. 9) in 17AA(-Arg-Glucose) medium to which had been added 2% of galactose and 2 μg. of arginine/ml. Fig. 9 (phase II) shows that the addition of 50 μg. of canavanine/ml. when the arginine was exhausted did not result in any further formation of β-galactosidase, despite the fact that the turbidity of the culture increased. Addition of 2 μg. of arginine/ml. in place of canavanine led to immediate formation of β-galactosidase.

Incorporation of [carboxy-^{14}C]*leucine.* The results reported above show that the increase in turbidity which occurred when cultures were incubated in the presence of canavanine was not associated with the formation of active forms of any of the enzymes tested. To decide whether protein synthesis occurred under these conditions, the uptake of [*carboxy*-^{14}C]-leucine into the protein + cell-wall fraction of the organisms was followed in the presence of an inhibitory concentration of canavanine. A culture was grown in the basal 17AA(-Arg) medium containing 2 μg. of arginine/ml. until the arginine was exhausted. The culture was then divided into four parts and the following additions were made: nil (culture 1); 2 μg. of arginine/ml. (culture 2); 100 μg. of arginine/ml. (culture 3); 50 μg. of canavanine/ml. (culture 4); [*carboxy*-^{14}C]leucine (final concn. mM) was added to each culture immediately after the addition of the amino acids. The cultures were incubated as described previously and measured samples, removed at intervals, were transferred to an equal volume of cold 10% trichloroacetic acid containing 1% (w/v) of [^{12}C]leucine to stop growth and incorporation. The rate of incorporation in the presence of the analogue into the protein + cell-wall fraction (Fig. 10*a*) was slower than that observed in arginine. The total amount of radioactivity incorporated in the canavanine experiment corresponds to about 5 μg.

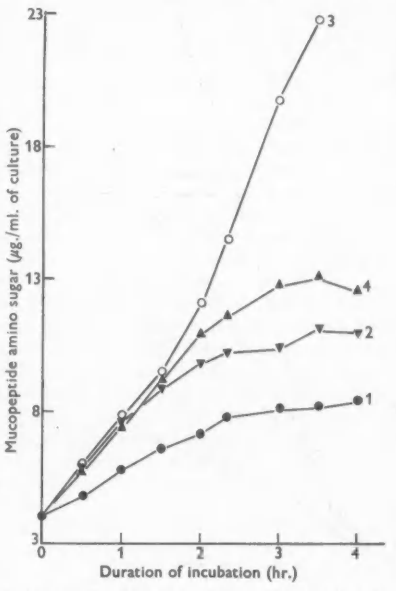

Fig. 8. Increase of 'mucopeptide' amino sugar in cultures incubated in the basal 17AA(-Arg) medium containing the following additions/ml.: 1 (●), nil; 2 (▼), 2 μg. of arginine; 3 (○), 100 μg. of arginine; 4 (▲), 50 μg. of canavanine. Initial culture density: 210 μg. dry wt. of organisms/ml.

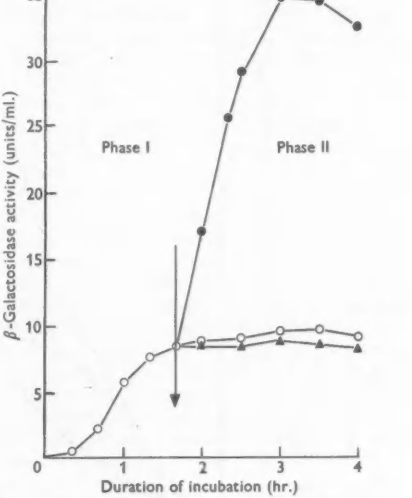

Fig. 9. Effect of arginine and canavanine on β-galactosidase formation in cultures partially induced by previous incubation in low concentrations of arginine. Media: phase I; 17AA(-Arg-Glucose) medium containing 2% of galactose and 2 μg. of arginine/ml. Further additions made at the arrow (phase II) were as follows: (●), 2 μg. of arginine/ml.; (○), nil; (▲), 50 μg. of canavanine/ml.

of leucine/mg. initial dry wt. of organisms. Addition of 30 μg. of chloramphenicol/ml. at the same time as the addition of the leucine resulted in a decrease of about 80% in the radioactivity incorporated into the protein + cell-wall fraction in the first 60 min. of incubation. Incorporation of radioactivity into the lipid fraction of the organisms occurred in the presence of canavanine (Fig. 10*b*). The rate of incorporation in the presence of canavanine was about the same as that occurring in the presence of arginine during the first 60 min. of incubation, and was unaffected by the presence of chloramphenicol.

DISCUSSION

Cultures of *Staphylococcus aureus* 524, incubated in media lacking arginine, are incapable of synthesizing RNA, DNA or protein. These changes are similar to those found by earlier workers (Sands & Roberts, 1952; Pardee & Prestidge, 1956; Yčas & Brawerman, 1957; Gros & Gros, 1958) with amino acid auxotrophs of *Escherichia coli* incubated in the absence of the required amino acid.

Despite the absence of protein synthesis the staphylococci were able to synthesize both lipid and the mucopeptide component of the cell wall, and it appears therefore that the omission from the growth medium of a single essential amino acid resulted in changes in cell biosynthesis very similar to those found when this strain was incubated in a buffered medium containing glucose and glycine (Rogers & Mandelstam, 1958; Mandelstam & Rogers, 1959). The use of trichloroacetic acid-precipitable amino sugar to estimate the level of the mucopeptide component of the cell wall is not vitiated by the recent findings of Baddiley and his co-workers (Armstrong, Baddiley, Buchanan & Carss, 1958; Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958) that the cell wall of several species of *S. aureus* contains a complex polymer of ribitol, *N*-acetyl glucosamine, D-alanine and orthophosphate, as this

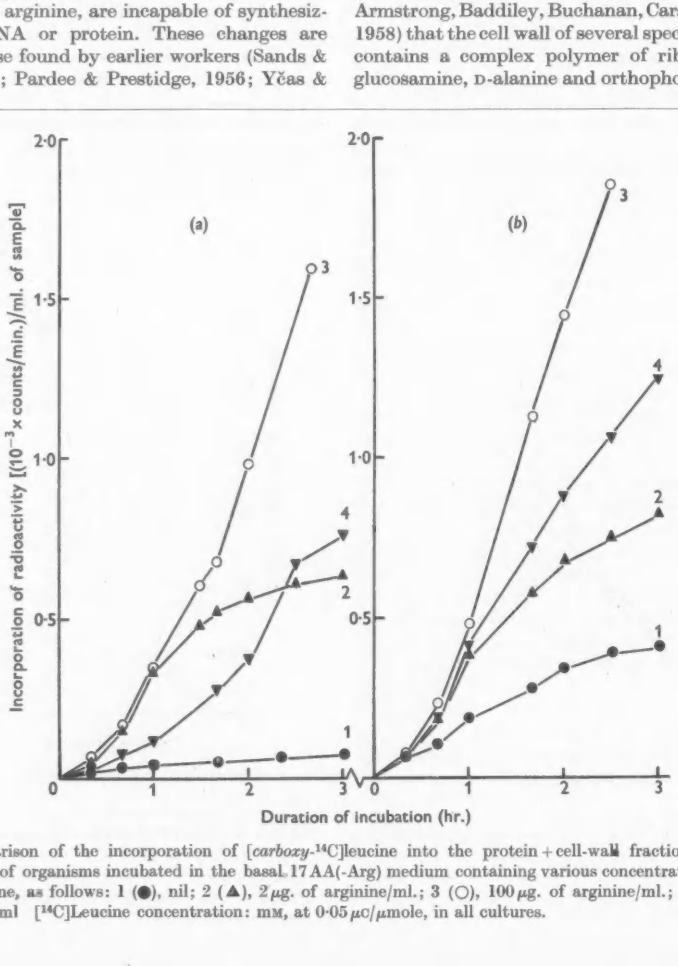

Fig. 10. Comparison of the incorporation of [*carboxy*-^{14}C]leucine into the protein + cell-wall fraction (*a*) and lipid fraction (*b*) of organisms incubated in the basal 17AA(-Arg) medium containing various concentrations of arginine or canavanine, as follows: 1 (●), nil; 2 (▲), 2 μg. of arginine/ml.; 3 (○), 100 μg. of arginine/ml.; 4 (▼), 50 μg. of canavanine/ml [^{14}C]Leucine concentration: mM, at 0·05 μC/μmole, in all cultures.

component may be extracted from cells with cold 5% trichloroacetic acid. Gros & Gros (1958), working with amino acid auxotrophs of *E. coli*, have shown that as little as 2 μg./ml. of the required amino acid allowed immediate synthesis of RNA. In the experiments reported above it was never possible to show an altered differential rate of RNA or DNA synthesis (ΔRNA/ΔG or ΔDNA/ΔG) on addition of low concentrations of arginine. These conditions did, however, have an effect on the synthesis of some of the enzymes studied. Although the differential rate of phosphatase synthesis was unaffected, no hyaluronidase was formed when cultures were incubated in the presence of 2 or 4 μg. of arginine/ml., and lytic-enzyme synthesis was delayed and occurred at a lower differential rate (see Fig. 5). These results show a range of sensitivity of the synthetic mechanism to low arginine concentration in this order: hyaluronidase > lytic enzyme > phosphatase, RNA, DNA > cell-wall mucopeptide. It was not possible to show any effect on the differential rates Δphosphatase/ΔRNA or Δphosphatase/ΔDNA, provided that the inorganic phosphate content of the growth medium was not altered. Similar experiments to study the differential rate of β-galactosidase formation showed a marked effect of arginine (Fig. 6), but these results are difficult to interpret for the following reasons. First, the estimation of β-galactosidase activity was carried out on intact organisms because disruption of the cells by any of the methods available led to complete loss of enzyme activity (cf. Creaser, 1955). The activity of the enzyme measured may not therefore reflect the amount of β-galactosidase protein synthesized but rather the activity of the system responsible for accumulating *o*-nitrophenyl-β-D-galactoside within the organisms (see Cohn, 1957).

Secondly, galactose is not a 'gratuitous' inducer (Monod & Cohn, 1952) of β-galactosidase for this strain of *S. aureus*, which seems to require carbohydrate in the culture medium for active growth and enzyme formation. Organisms grown in glucose did not contain an active 'galactozymase' system and could not therefore metabolize galactose in large quantities to produce energy. Furthermore, glucose could not be added with the galactose as it completely suppresses the formation of both the 'galactozymase' system and β-galactosidase. On transfer from glucose- to galactose-containing medium the 'galactozymase' system was induced (Creaser, 1955; M. H. Richmond, unpublished work), and this breaks down the galactose to produce the energy necessary for rapid growth and β-galactosidase synthesis. It follows therefore that the addition of galactose to cultures resulted in several changes in the metabolism of the organisms and these may secondarily affect the synthesis of β-galactosidase.

If this difficulty is ignored, the sequence of decreasing sensitivity of synthetic processes to low arginine concentration becomes: hyaluronidase > lytic enzyme > β-galactosidase > phosphatase, RNA, DNA > cell-wall synthesis.

The studies reported by Virtanen (1948; Virtanen & De Ley, 1948) on enzyme formation in *E. coli* grown on very low concentrations of ammonium ion as sole nitrogen source, could be consistent with these results. However, these authors studied total enzyme content of the organisms and not the rates of enzyme formation.

Both hyaluronidase and the lytic enzyme are predominantly extracellular and this may be related to their particular sensitivity to arginine deprival. Gale (1953) has reported that *S. aureus* is capable of accumulating amino acids within the osmotic membrane of the cell. In this way the concentration of a limiting amino acid within the cell may be about 10–20 times its concentration in the growth medium. Experiments with protoplasts of Gram-positive bacteria have led to the view that the osmotic barrier of the Gram-positive cell is in the cytoplasmic membrane (Weibull, 1956; McQuillen, 1956, 1958) and, as more than 85% of the total extractable amounts of both hyaluronidase and the lytic enzyme may be detected in the growth medium, it seems likely that these enzymes are synthesized in this membrane. If this is so, the synthetic sites of these enzymes may not be supplied with arginine that has passed through the osmotic barrier/cytoplasmic membrane, but directly from the growth medium. In this way, when a limited quantity of arginine is available, the level of amino acid reaching the hyaluronidase- and lytic enzyme-synthesizing sites may be appreciably lower than that reaching the enzyme-forming sites within the organisms. In non-limiting concentrations of arginine this effect would be lessened, and this is supported by the finding that synthesis of both extracellular enzymes is rapid in the presence of 100 μg. of arginine/ml. This mechanism cannot, however, be the direct cause of the increased differential rate of hyaluronidase and lytic-enzyme synthesis observed as exponential growth proceeds (Rogers, 1954, 1957; Richmond, 1959*a*).

Organisms grown in media in which arginine was replaced by canavanine were able to incorporate radioactive leucine into the protein fraction of the organisms, but formed no active phosphatase, β-galactosidase, hyaluronidase or lytic enzyme. The incorporation was inhibited more than 80% by concentrations of chloramphenicol inhibitory to growth, and these results imply that protein synthesis continued in these cultures for about 2–3 hr. but without leading to the formation of active

versions of the enzyme tested. Munier & Cohen (1956) have shown in similar experiments, involving *p*-fluorophenylalanine, that the analogue was incorporated into the cell protein in place of phenylalanine. Canavanine has been reported to replace arginine in a number of biosynthetic reactions (Nakatsu, 1956; Walker, 1956; Kihara & Snell, 1957), and it seemed likely that the protein synthesis occurring in cultures grown in the presence of the analogue was associated with incorporation of canavanine into protein in the place of arginine. The detection of canavanine in the protein fraction of the organisms (M. H. Richmond, unpublished work) supports this hypothesis. It is not possible to say at this juncture whether canavanine inhibits the formation of phosphatase, β-galactosidase, hyaluronidase and the lytic enzyme completely or whether the formation of inactive enzyme 'analogues' occurs. If modified enzyme molecules are made, the effect of the amino acid analogues on their activity would depend, chiefly, on two factors. First, whether the analogue completely replaces the normal amino acid. Secondly, whether the replacement of a given amino acid residue results in an enzyme with a modified activity, or an inactive protein.

In the experiments reported above, the organisms are completely exacting to arginine and therefore, if enzyme 'analogues' are formed, their arginine residues would probably be completely replaced with canavanine and be inactive. Experiments to decide whether phosphatase, β-galactosidase, hyaluronidase or lytic-enzyme 'analogues' are formed under the conditions of these experiments must await methods of purification of the enzymes and the preparation of specific antisera.

SUMMARY

1. Cultures of *Staphylococcus aureus* 524, incubated in a fully synthetic amino acid medium lacking arginine, synthesize some lipid and the mucopeptide component of the bacterial cell wall but no ribonucleic acid, deoxyribonucleic acid and general cell protein. Similarly, such cultures do not form active extracellular hyaluronidase, lytic enzyme and intracellular phosphatase; nor can they be induced to form intracellular β-galactosidase.

2. Addition of 2 μg. of arginine/ml. to such cultures fully restores the ability of the organisms to synthesize ribonucleic acid, deoxyribonucleic acid and phosphatase. Under these conditions, however, no hyaluronidase formation can be detected, the synthesis of lytic enzyme is markedly impaired and the differential rate of β-galactosidase formation is depressed to about half the rate achieved in 100 μg. of arginine/ml.

3. As a result of these experiments, the following sequence of decreasing sensitivity to limiting arginine concentrations is suggested: hyaluronidase > lytic enzyme > β-galactosidase > phosphatase, ribonucleic acid and deoxyribonucleic acid > cell-wall mucopeptide.

4. Cultures incubated in a fully synthetic amino acid medium containing canavanine in the place of arginine increase in turbidity, synthesize cell-wall mucopeptide, ribonucleic acid, deoxyribonucleic acid and general cell protein but do not form active versions of any of the enzymes tested.

5. A possible mechanism of these changes is discussed.

I should like to express my gratitude to Dr M. R. Pollock, Dr H. J. Rogers and my colleagues in Bacteriology Division, National Institute for Medical Research, for much stimulating discussion in connexion with this work; and to Drs Rogers and Mandelstam for a preview of their paper (Mandelstam & Rogers, 1959).

REFERENCES

Armstrong, J. J., Baddiley, J., Buchanan, J. G. & Carss, B. (1958). *Nature, Lond.*, **181**, 1692.
Armstrong, J. J., Baddiley, J., Buchanan, J. G., Carss, B. & Greenberg, G. R. (1958). *J. chem. Soc.* p. 4344.
Brawerman, G. & Yčas, M. (1957). *Arch. Biochem. Biophys.* **68**, 112.
Burton, K. (1955). *Biochem. J.* **61**, 473.
Cohn, M. (1957). *Bact. Rev.* **21**, 140.
Cowie, D. & Cohen, G. N. (1957). *Biochim. biophys. Acta*, **26**, 252.
Creaser, E. H. (1955). *J. gen. Microbiol.* **12**, 288.
Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
Gale, E. F. (1953). *Advanc. Protein Chem.* **8**, 285.
Gale, E. F. & Folkes, J. (1953). *Biochem. J.* **55**, 721.
Gale, E. F. & Folkes, J. (1955). *Biochem. J.* **59**, 661.
Gros, F. & Gros, F. (1958). *Exp. Cell Res.* **14**, 104.
Hancock, R. & Park, J. T. (1958). *Nature, Lond.*, **181**, 1050.
Hogness, D. H., Cohn, M. & Monod, J. (1955). *Biochim. biophys. Acta*, **16**, 99.
Horowitz, N. H. & Srb, A. M. (1948). *J. biol. Chem.* **174**, 371.
Hughes, D. (1951). *Brit. J. exp. Path.* **32**, 97.
Kantorowicz, O. (1951). *J. gen. Microbiol.* **5**, 276.
Kihara, H. & Snell, E. E. (1957). *J. biol. Chem.* **226**, 485.
Kitagawa, M. & Yamada, H. (1932). *J. Biochem., Tokyo*, **16**, 399.
McQuillen, K. (1956). *Symp. Soc. gen. Microbiol.* **6**, 127.
McQuillen, K. (1958). *J. gen. Microbiol.* **18**, 498.
McQuillen, K. & Roberts, R. B. (1954). *J. biol. Chem.* **207**, 81.
Mandelstam, J. & Rogers, H. J. (1959). *Biochem. J.* **72**, 654.
Mickle, H. (1948). *J. R. micr. Soc.* **68**, 10.
Monod, J. (1942). *Recherches sur la croissance des cultures bactériennes*. Paris: Hermann et Cie.
Monod, J. (1956). In *Enzymes: Units of Biological Structure and Function*, p. 7. Ed. by Gaebler, O. H. New York: Academic Press Inc.

Monod, J., Cohen-Bazire, G. & Cohn, M. (1951). *Biochim. biophys. Acta*, **7**, 585.
Monod, J. & Cohn, M. (1952). *Advanc. Enzymol.* **13**, 67.
Monod, J., Pappenheimer, A. M., jun. & Cohen-Bazire, G. (1952). *Biochim. biophys. Acta*, **9**, 648.
Munier, R. L. & Cohen, G. N. (1956). *Biochim. biophys. Acta*, **21**, 592.
Nakatsu, S. (1956). *J. Biochem., Tokyo*, **43**, 675.
Ogur, M. & Rosen, G. (1950). *Arch. Biochem.* **25**, 262.
Pardee, A. B. & Prestidge, L. (1956). *J. Bact.* **71**, 677.
Pardee, A. B. & Prestidge, L. (1958). *Biochim. biophys. Acta*, **27**, 330.
Pardee, A. B., Shore, V. G. & Prestidge, L. (1956). *Biochem. biophys. Acta*, **21**, 406.
Perkins, H. R. & Rogers, H. J. (1959). *Biochem. J.* **72**, 647
Pollock, M. R. & Kramer, M. (1958). *Biochem. J.* **70**, 665.
Richmond, M. H. (1958). *J. gen. Microbiol.* **18**, xii.
Richmond, M. H. (1959*a*). *Biochim. biophys. Acta*, **31**, 564.
Richmond, M. H. (1959*b*). *Biochim. biophys. Acta*, **33**, 78.
Rickenberg, H. V. & Lester, R. (1955). *J. gen. Microbiol.* **13**, 279.
Rogers, H. J. (1945). *Biochem. J.* **39**, 435.
Rogers, H. J. (1953). *J. Path. Bact.* **66**, 545.
Rogers, H. J. (1954). *J. gen. Microbiol.* **10**, 209.
Rogers, H. J. (1957). *J. gen. Microbiol.* **16**, 22.
Rogers, H. J. & Mandelstam, J. (1958). *Nature, Lond.*, **181**, 956.
Salton, M. R. J. (1951). *J. gen. Microbiol.* **5**, 391.
Sands, M. K. & Roberts, R. B. (1952). *J. Bacteriol.* **63**, 505.
Szafir, J. J. & Bennett, E. O. (1953). *Science*, **117**, 717.
Virtanen, A. I. (1948). *Svensk kem. Tidskr.* **60**, 23.
Virtanen, A. I. & De Ley, J. L. (1948). *Arch. Biochem.* **16**, 169.
Volcani, E. B. & Snell, E. E. (1948). *J. biol. Chem.* **174**, 893.
Walker, J. B. (1955). *J. biol. Chem.* **212**, 207.
Walker, J. B. (1956). *J. biol. Chem.* **218**, 569.
Weibull, C. (1956). *Symp. Soc. gen. Microbiol.* **6**, 111.
Yčas, M. & Brawerman, G. (1957). *Arch. Biochem. Biophys* **68**, 118.

The Spectrophotometric Determination of Protein at 210 mμ

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The absorption spectra of proteins in the region above 240 mμ are well known and extensively used in analytical procedures. The peak in the region of 280 mμ may be used for the analysis of tyrosine and tryptophan (Beaven & Holiday, 1952), and measurement of this peak provides, for relatively homogeneous proteins, a simple and accurate method of determining relative concentrations (e.g. Tombs, 1957). With more complex mixtures such as serum the various proteins will contain widely varying amounts of tyrosine, tryptophan and phenylalanine, and measurements at 280 mμ can no longer be used as a reliable index of total protein content, even where a comparison between one serum and another is all that is required.

Below 230 mμ the absorption of both proteins and peptides rises steeply. However, Goldfarb, Saidel & Mosovich (1951) have shown that there is a definite maximum near 190 mμ which is due mainly to the specific absorption of peptide bonds. If this is so one might expect that all proteins would have a similar specific absorption in this region, since they all have similar peptide-bond contents. Goldfarb *et al.* (1951) report figures which suggest that this is indeed the case for human and bovine albumin and γ-globulin, egg albumin and gelatin. It seemed possible therefore that measurement in the neighbourhood of this peak might offer a rapid and simple method for the estimation of proteins.

A method involving measurement at 215 and 225 mμ has recently been proposed by Waddell (1956) and Bendixen (1957). The choice of a suitable wavelength in this region depends partly on instrumental limitations and partly on the possible contribution of the aromatic amino acids to the specific absorption of individual proteins. Results described here and briefly reported elsewhere (Tombs, Souter & Maclagan, 1959) suggest that 210 mμ is the optimum wavelength for our conditions of measurement.

EXPERIMENTAL AND RESULTS

Nitrogen estimations. Total protein from 0·1 ml. of serum was precipitated with aluminium tungstate (Lorant, 1957), and nitrogen in the precipitate was determined by the micro-Kjeldahl method. A factor of 6·25 was used to convert nitrogen content into protein content.

Absorption measurements. These were made with a standard Hilger Uvispek spectrophotometer, but similar results have also been obtained with a Unicam SP. 500 spectrophotometer. Paired 1 cm. silica cells were used with solvent as blank. A slit width of 1·4 mm. was used at 210 mμ. Cells were cleaned in conc. HNO_3 and cell-matching was checked at frequent intervals.

Solvents. Measurements made in water, 0·9% NaCl soln. and phosphate buffers were identical, but 0·9% NaCl was generally adopted to avoid the possibility of precipitating abnormal globulins. Gramicidin, however, had to be dissolved in absolute ethanol, and its absorption was measured against ethanol as blank; the absorption of absolute ethanol at 210 mμ was sufficiently low (0·45) to permit its use as a solvent.

Collection and storage of serum. Serum samples were taken from those used for normal routine laboratory estimations. They were either used on the day of collection or stored in the frozen state at −15°; repeated freezing and thawing had no effect on the absorption at 210 mμ.

Previous work suggested that 205 mμ might be the wavelength of choice, but with our instrument it was found that 210 mμ was the lowest practicable. Table 1 shows some data which, although not exhaustive, indicate that at this wavelength a considerable variety of serum proteins have specific extinctions close to 200 although there are wide differences between their specific extinctions at 280 mμ.

Gramicidin (40% of tryptophan) shows much greater absorption at both wavelengths, and it is evident for reasons given below that this feature would be still more evident at wavelengths longer than 210 mμ. Subsequent measurements were therefore made at this point.

Instrumental limitations. Measurements made on the same instrument over a period of several months showed a steadily increasing degree of deviation from linearity, particularly at extinctions over 1·0 (Fig. 1), and a marked reduction in absorption at wavelengths below 210 mμ (Fig. 2). These effects are characteristic of stray radiation, accentuated by deterioration of optical components and ageing of the lamp (Scott, 1955). Although correction for stray radiation can be made it is laborious for a routine method. It is preferable to use the instrument in conditions where stray radiation is unimportant, which can be done by cleaning the optical faces and replacing the lamp as soon as any deviation from Beer's Law is noted. We have found it essential to employ the two standards described below with each batch of measurements to ensure that a linear response is obtained, and it is desirable to avoid the use of extinction values over 1·0.

Reference standard. Any known protein solution will serve as a suitable standard. We have found bovine albumin (6·0 g./100 ml.) to be suitable. Two dilutions of 1/1500 and 1/3000 (v/v) were

Table 1. *Specific absorption of some typical serum proteins*

Protein preparation	$E^{1\%}_{1\,cm.}$ 280 mμ	210 mμ
Total serum proteins	About 10	205 (±1·4)
Human albumin	6	203
Human γ-globulin	15	213
Human siderophilin	14	200
Mixed α- and β-globulin	13	214
Bovine albumin	6·8	204
Gramicidin	62	342

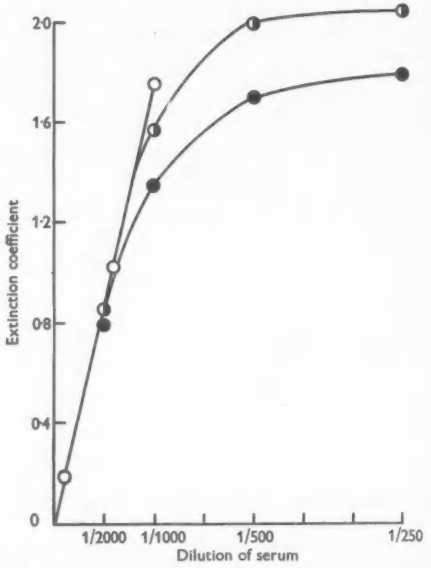

Fig. 1. Deviations from Beer's Law due to ageing of the hydrogen lamp. ○, New lamp; ●, same lamp after about 600 hr. use; ◑, reactivated lamp.

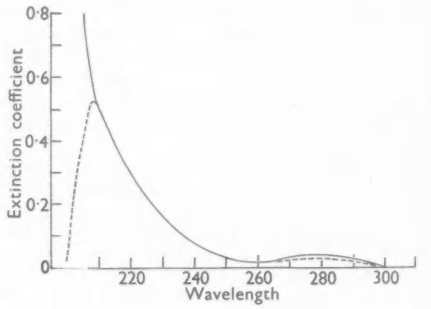

Fig. 2. Absorption spectrum of 1/1000 dilutions of two sera showing the effect of using an aged hydrogen lamp. —, New lamp; - - - -, aged lamp (approx. 600 hr.). The curve measured with the new lamp has been adjusted so as to coincide with the other over the range 210–240 mμ.

used for reasons given below. The stock solution remained stable for at least a week at 4°.

Procedure for total serum-protein determination. Serum (0·1 ml.) was diluted to 200 ml. with 0·9% NaCl soln. and the extinction was measured at 210 mμ within 2 hr. of dilution.

Results were considered acceptable if twice the low standard reading was within 3% of that given by the high standard. Total serum protein was then calculated by simple proportionality from the serum and standard readings.

Serum proteins. Isolated fractions were obtained by column chromatography on cellulose derivatives (Sober, Gutter, Wyckoff & Peterson, 1956) by unpublished methods.

The specific extinction of the total proteins of 40 normal and pathological sera at 210 mμ, $E^{1\%}_{1\,cm.}$ was 205 ± 1·4 (S.E.), range 194–226. This was based on Kjeldahl nitrogen determinations and agrees well with Goldfarb *et al.* (1951), who found for human γ-globulin 208 and for albumin 201, determined on a weight basis. The conclusion that the majority of serum proteins have closely similar specific extinctions at 210 mμ was reinforced by the results given in Fig. 3, which show a linear relationship between extinction at 210 mμ and protein nitrogen in 26 miscellaneous sera. Linearity in the extinction range 0–1·7 was checked with more dilute protein solutions. Although the results shown in Fig. 3 were measured at 1/1000 dilution of serum and the instrument was satisfactory at this time, it was subsequently found that deterioration of the lamp made values measured at a 1/1000 dilution more liable to error than at a 1/2000 dilution. For this reason a 1/2000 dilution of serum was adopted as standard.

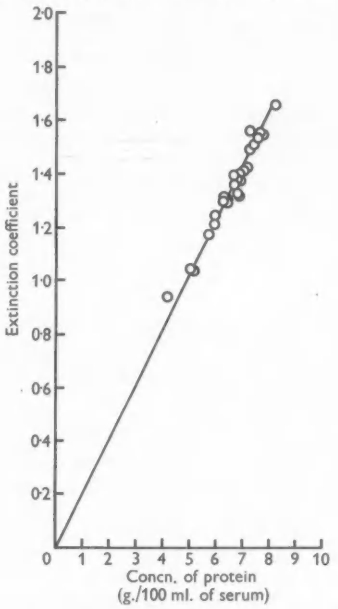

Fig. 3. Total serum protein determined by Kjeldahl analysis, plotted against the extinction coefficient of a 1/1000 dilution of serum, in 26 samples.

Measurement of sera taken from the same individual before and after feeding showed no detectable effect of ordinary degrees of lipaemia. In one individual there was an apparent increase of 0·3 g. of protein/100 ml. of serum after fat-feeding, but this serum showed an exceptional degree of lipaemia. pH variation between 2 and 10 had little effect on absorption. The extinction of 1/1000 or 1/2000 dilutions of serum remained constant for 2 hr., and then rose by about 2% over the next 4 hr. It is therefore important to make measurements within 2 hr. of dilution.

Albumin determination. Half-saturation with $(NH_4)SO_4$ was employed as follows: 0·1 ml. of serum was added to 4·9 ml. of $(NH_4)_2SO_4$ solution (2·09 M; 276 g./l.). After centrifuging in an angle-head centrifuge at 6000 *g* for 10 min., albumin was estimated on a suitable dilution of the supernatant fluid (usually 0·5 ml. + 9·5 ml. of water). Alternatively, globulins were precipitated by 21% (w/v) Na_2SO_4 (Howe, 1921) and albumin was estimated in the filtrate.

In the determination of albumin:globulin ratios $(NH_4)_2SO_4$ is preferable to Na_2SO_4 for the precipitation of globulins because the mixture can be centrifuged at room temperature. Table 2 shows reasonable agreement between the results obtained when $(NH_4)_2SO_4$ and Na_2SO_4 are used for precipitation, followed by spectrometric estimation of the albumin. Table 3 shows the results of spectrometric estimations with $(NH_4)_2SO_4$ used for precipitation, and routine laboratory measurements with Na_2SO_4 and micro-Kjeldahl nitrogen estimations. The agreement was not exact but was within the reproducibility normally found for this determination, and the two methods showed no systematic difference.

DISCUSSION

The wavelength chosen for this work appears to be better than those previously employed because it diminishes greatly the variations in specific extinction between different proteins. This is especially true of serum proteins since all the examples which we have tested showed closely similar specific extinctions.

There is a large number of possible interfering factors since all carboxylic acid and buffer ions,

alcohols, bicarbonate and aromatic compounds absorb in this region. However, with serum the overwhelming majority of absorbing material is the protein and this, coupled with the very high specific extinction of proteins in this region, reduces the contribution to the absorption from substances other than protein to negligible levels. This was shown by the fact that the absorption of protein-free filtrates of serum prepared by the zinc hydroxide precipitation method was approximately 0·5 % of that of the original serum. (Values up to 0·6 % were, however, obtained with citrated bank plasma, owing to the high level of citrate.) Although a similar method has been successfully employed on cerebrospinal fluid (Waddell, 1956), it cannot be used on urine where the levels of interfering substances are much higher. There are also severe restrictions on permissible solvents, e.g. buffers containing carboxylic acids cannot be used.

Although a large part of the absorption at 210 mμ is due specifically to peptide bonds, the aromatic amino acids, particularly tryptophan, also make an appreciable contribution (approximately one-quarter of the total for human albumin), and it was to be expected that proteins rich in these amino acids would have higher specific extinctions than others. Thus Goldfarb *et al.* (1951) found $E^{1\%}_{1\,cm.}$ for insulin (12 % of tyrosine, 8 % of phenylalanine) to be about 250. A more extreme case is that of gramicidin, containing about 40 % of tryphophan, for which we found a specific extinction of 340 (average for serum proteins 205).

The absorption spectrum of tryptophan rises steeply below 230 mμ to a maximum at 220 mμ, shows a minimum at 205 mμ and then rises steeply again (Saidel, 1955), whereas the curve for proteins shows no minimum in this region. Tyrosine and phenylalanine behave similarly to tryptophan although their specific absorptions are lower. Values around 220 mμ are therefore undesirable, and 205 mμ is the wavelength at which the aromatic amino acids make the least contribution to the total absorption. Gramicidin shows a maximum at 225 mμ, no doubt due to its tryptophan content. For technical reasons (see Experimental and Results), 210 mμ was the lowest wavelength at which we could operate consistently and appears to be the optimum value for the instruments employed. Further, the specific extinction of tryptophan is the same at 210 mμ and about 227 mμ, so that differential measurements near these two wavelengths could be used in principle to eliminate the contribution of tryptophan completely. Waddell (1956) has described a method

Table 2. *Comparison of ammonium sulphate* (2·05 M) *and sodium sulphate* (1·47 M) *for globulin precipitation*

Estimations were made by the spectrometric method (see text). A/G, Albumin:globulin ratio.

	Pptn. with 2·05 M-$(NH_4)_2SO_4$			Pptn. with 1·47 M-Na_2SO_4		
Serum	Albumin (g./100 ml.)	Globulin (g./100 ml.)	A/G	Albumin (g./100 ml.)	Globulin (g./100 ml.)	A/G
1	4·1	2·5	1·7	4·1	2·5	1·7
2	3·7	2·5	1·5	4·2	2·0	2·1
3	2·6	2·2	1·2	2·8	2·0	1·4
4	4·4	3·3	1·3	4·3	3·5	1·2
5	6·1	1·3	4·7	5·4	1·4	3·9
6	3·4	2·6	1·3	3·1	2·8	1·1
7	1·9	2·8	0·7	1·9	2·8	0·7
8	4·4	2·6	1·7	4·9	2·2	2·1
9	3·5	3·0	1·1	3·7	2·8	1·3
10	4·4	3·9	1·1	4·4	3·8	1·1

Table 3. *Comparison of albumin: globulin (A/G) ratios by Kjeldahl (sodium sulphate) and spectroscopic (ammonium sulphate) methods*

Serum	Albumin (Kjeldahl) (g./100 ml.)	Albumin (spect.) (g./100 ml.)	Globulin (Kjeldahl) (g./100 ml.)	Globulin (spect.) (g./100 ml.)	A/G (Kjeldahl)	A/G (spect.)
1	3·2	2·8	3·1	3·2	1·0	0·9
2	3·3	2·8	5·3	3·9	0·6	0·7
3	3·5	3·5	3·6	3·2	1·0	1·1
4	5·8	6·1	1·6	1·3	3·5	4·7
5	4·4	4·1	3·0	2·5	1·5	1·7
6	2·2	2·3	1·9	2·4	1·1	1·1
7	4·3	4·4	3·5	3·9	1·2	1·1
8	4·7	4·4	3·1	3·3	1·5	1·3
9	4·7	4·6	2·6	2·1	1·8	2·2
10	3·3	3·0	2·6	2·9	1·2	1·0
11	1·9	1·9	3·3	2·8	0·6	0·65

based on the peptide-bond absorption, differential measurements being used in which the extinction at 225 mμ is subtracted from that at 215. The use of these wavelengths would not, however, have the effect of reducing the contribution of the aromatic amino acids, and cannot be applied to cases such as gramicidin, since this peptide actually has a greater absorption at 225 than at 215 mμ.

From our results it seems that measurement in the region of 210 mμ can be used as a reliable, precise and quick method for the estimation of serum proteins. The use of measurements at this wavelength also appears to provide a fairly general method of protein estimation, and serious error is unlikely unless the protein contains exceptionally large amounts of aromatic amino acids, as with gramicidin or insulin.

A further advantage of this method lies in the great sensitivity. When no interfering substances were present it was possible to detect concentrations down to 2 μg. of protein/ml., corresponding to an extinction of 0·04. Advantage was taken of this in experiments with anion-exchange cellulose for the column chromatography of serum proteins. Amounts of serum as low as 0·1 ml. were used satisfactorily and the quantitative interpretation of results was aided by the uniformity of specific absorption in the various fractions.

SUMMARY

1. Absorption measurements at 210 mμ can be usefully employed for protein estimation. At this wavelength the total proteins of 40 normal and pathological sera had a mean specific extinction coefficient $E^{0.1\%}_{1\,cm.}$ of 20·5 ± 0·14. Albumin, α-, β- and γ-globulin all had specific extinction $E^{0.1\%}_{1\,cm.}$ near 20. The method is directly applicable to all proteins studied except those containing unusually large amounts of aromatic amino acids.

2. A method for the estimation of total serum proteins and for albumin:globulin ratios based on the above is described. Bovine serum albumin had almost the same specific extinction coefficient as serum proteins and formed a useful reference standard.

3. The use of the extinction at 210 mμ for the detection of very small quantities of protein (2 μg./ml.) and its application to column chromatography are discussed. In this connexion it is necessary to avoid the use of buffers containing carboxylic acids.

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REFERENCES

Beaven, G. H. & Holiday, E. R. (1952). *Advanc. Protein Chem.* **7**, 352.
Bendixen, G. (1957). *Nord. med. Tidskr.* **58**, 1487.
Goldfarb, A. R., Saidel, L. J. & Mosovich, E. (1951). *J. biol. Chem.* **193**, 397.
Howe, J. (1921). *J. biol. Chem.* **49**, 109.
Lorant, I. St. (1957). *J. clin. Path.* **10**, 136.
Saidel, L. J. (1955). *Arch. Biochem. Biophys.* **54**, 184.
Scott, J. F. (1955). *Physical Techniques in Biological Research.* New York: Academic Press Inc.
Sober, H. A., Gutter, F. J., Wyckoff, M. M. & Peterson, E. A. (1956). *J. Amer. chem. Soc.* **78**, 756.
Tombs, M. P. (1957). *Biochem. J.* **67**, 517.
Tombs, M. P., Souter, F. & Maclagan, N. F. (1959). *Biochem. J.* **71**, 13P.
Waddell, W. J. (1956). *J. Lab. clin. Med.* **48**, 311.

Some Properties of Uterus Actomyosin and Myofilaments

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Some properties of the actomyosin-like adenosine triphosphatase of the uterus have already been described (see Needham & Cawkwell, 1956). It was shown that this enzyme splits off only one phosphate group and has a pH–activity curve in 0·5M-potassium chloride resembling that of myosin from skeletal muscle. The activity, however, is much lower than that of skeletal-muscle actomyosin; rabbit- and pig-uterus actomyosin (with which most of this work was done) also differ from skeletal muscle actomyosin (which is somewhat inhibited at higher concentrations of potassium chloride) in being less active in media containing 0·1M-potassium chloride than in those containing 0·5M-potassium chloride. Thus at 28°, with Ca^{2+} ion activation, the values for μmoles of inorganic phosphorus liberated/mg. of nitrogen/min. are about 0·2 and 0·6 in media containing 0·1 and

0·5 M-potassium chloride respectively; the corresponding values for skeletal-muscle actomyosin are about 6·0 and 3·6.

In the presence of Mg^{2+} ions skeletal-muscle actomyosin has an activity of about 3 μmoles/mg. of nitrogen/min., under optimum conditions (calculated for 28° from the data at 20° of Hasselbach, 1952). Uterus actomyosin had negligible activity with Mg^{2+} ions under the same conditions and a variety of others tested.

In the present work the further purification of uterus actomyosin is described and the action upon it of crystalline trypsin under conditions closely resembling those used with skeletal-muscle myosin (Szent-Györgyi, 1953). In the latter case a decrease of 13% was found in total adenosine triphosphatase activity by Mihalyi & Szent-Györgyi (1953*b*), whereas Gergely, Gouvea & Karibian (1955) report an increase of about 25%. The myosin is split into two components, heavy and light meromyosin (H- and L-meromyosin), the former constituting a little more than 50% of the protein; it contains all the adenosine triphosphatase activity and power of complex formation with actin. Skeletal-muscle actomyosin is digested at the same rate as myosin; in this case L-meromyosin is released, but H-meromyosin is obtained in combination with actin (Szent-Györgyi, 1953; Mihalyi & Szent-Györgyi, 1953*b*).

With uterus actomyosin, there is a striking rise in total adenosine triphosphatase activity upon treatment with trypsin: 400–800% tested in 0·1 M-potassium chloride and about 50% tested in 0·5 M-potassium chloride; moreover, a fraction could be obtained corresponding to the acto-H-meromyosin of skeletal muscle, with adenosine triphosphatase activity as high as that of the latter.

This high potential adenosine triphosphatase activity of the uterus protein led us to examine various possibilities of partial inactivation during preparation of the enzyme from the native smooth-muscle filaments.

Snellman & Tenow (1954) have claimed that the uterine contractile mechanism contains actotropomyosin instead of actomyosin, and suggest that the former consists of a complex of actin, a 'phosphate-absorbing protein' and nucleotropomyosin. In view of this we have estimated tropomyosin in pig uterus and also in the usual actomyosin precipitates.

A preliminary account of some of this work has been published (Needham & Cawkwell, 1958).

EXPERIMENTAL

Materials

Preparation of the purified actomyosin. The early stages were similar to those described by Needham & Cawkwell (1956), except that when small quantities of tissue were used (20–30 g.) it was disintegrated at 4° by thorough grinding with acid-washed sand or quartz, the extracting fluid being added gradually. This had the advantage over the use of the Waring Blendor that frothing was avoided. After two precipitations at 0·04 M-KCl, three further precipitations were carried out; the final concentration of KCl in each case was adjusted to 0·25 M, and the pH to 6·85 with the use of 0·66 M-glyoxaline–HCl buffer.

Cell-particle suspension. This was made as described by Needham & Cawkwell (1956). The residues obtained after centrifuging the 0·5 M-KCl extract at 100 000 *g* always

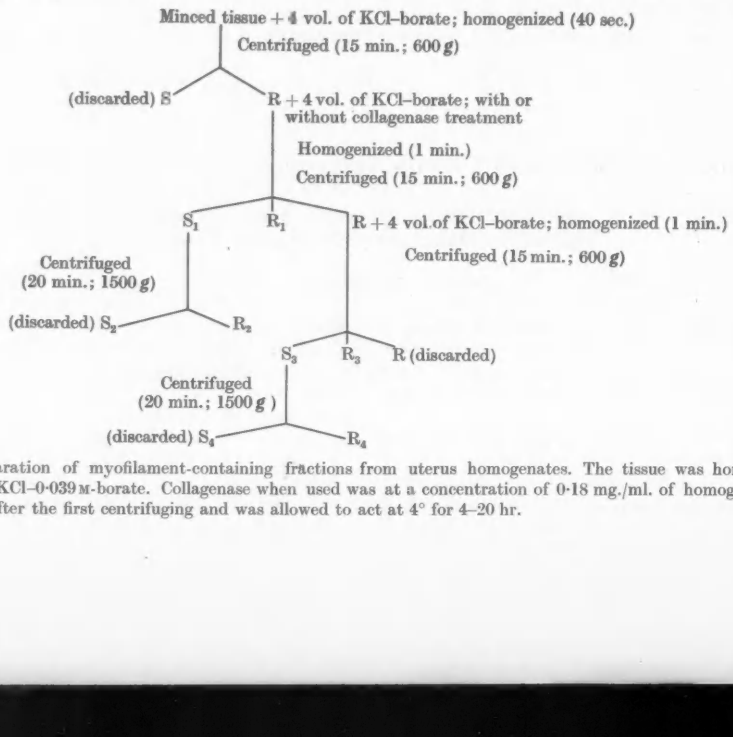

Fig. 1. Separation of myofilament-containing fractions from uterus homogenates. The tissue was homogenized in 0·025 M-KCl–0·039 M-borate. Collagenase when used was at a concentration of 0·18 mg./ml. of homogenate; it was added after the first centrifuging and was allowed to act at 4° for 4–20 hr.

showed a transparent, jelly-like uppermost layer, probably consisting of a little deposited actomyosin; to free the particles from this, the suspension made as described in 0·5 M-KCl containing 0·05 M-potassium phosphate (pH 7·5) was allowed to stand for a few hours and then centrifuged again and the sediment resuspended. Before use in the tests for adenosine triphosphatase activity it was dialysed for some hours against 0·5 M-KCl.

Preparation of myofilament-containing fractions. Homogenization of the minced tissue in 0·039 M-borate buffer (pH 7·0) containing 0·025 M-KCl was carried out at 4° in a Waring Blendor for large quantities of pig uterus, or in a small plastic blendor (about 40 ml. capacity) for rabbit uterus. The latter had the advantage that frothing could be almost completely avoided; the tissue used with it was first passed through a Latapie mincer. The medium used was that found by Perry & Grey (1956) to give least leaching out of myofibrillar protein in skeletal muscle. Several trials led to the adoption of the procedure shown in Fig. 1. All values given for *g* are average values.

The collagenase treatment mentioned in Fig. 1 consisted in adding collagenase (0·18 mg./ml.) to the homogenate and keeping it for different periods (4–20 hr.) at 4°.

Enzymes. The crystalline trypsin used was the salt-free product of the Novo Terapeutisk Laboratorium, Copenhagen. The trypsin inhibitor (made from soya bean and crystallized from ethanol) was from the Worthington Biochemical Corp., Freehold, N.J., U.S.A.

The collagenase used was ABC Form II, from the Agricultural Biologicals Corp., Lynbrook, N.Y., U.S.A. It is described as containing only traces of other proteinase.

Special chemicals. The adenosine triphosphate (ATP) used was the sodium salt supplied by the Sigma Chemical Co., St Louis, Mo., U.S.A.

Glutathione was obtained from the National Biochemical Corp., Cleveland, Ohio, U.S.A. Solutions were made up and neutralized immediately before the experiment.

2:3-Dimercaptopropanol (BAL) was the gift of Sir Rudolph Peters. It was obtained as a saturated solution in water by allowing the oil to stand covered by a few millilitres of water for some days with occasional shaking. The solution was standardized by titration against 0·01 N-iodine and neutralized just before use.

Di*iso*propyl phosphorofluoridate (DFP) was the gift of Dr B. C. Saunders to Dr M. Dixon. It was used as a solution (0·1 M) in propan-2-ol.

Buffers. Phosphate buffers were prepared from KH_2PO_4 (analytical reagent, Hopkin and Williams Ltd.) brought to the desired pH by means of KOH solution. Glyoxaline was supplied by L. Light and Co. Ltd.; if not completely colourless it was recrystallized first from benzene and then from ethanol. Buffer solutions were made by addition of the required amounts of N-HCl. Borate buffers about pH 7·0 were made from appropriate mixtures of 0·05 M-sodium tetraborate and 0·2 M-boric acid (Palitzsch, 1915; Britton, 1955); for higher pH values mixtures of 0·2 N-NaOH and 0·2 M-boric acid were used (Britton, 1955). All pH values given for buffers or buffered solutions were measured by means of the glass electrode.

Methods

Estimation of tropomyosin. The assay method of Bailey (1948) was carried out on pig myometrium for the quantitative separation of tropomyosin. Tropomyosin was also estimated in an actomyosin preparation from pig myometrium. The preparation was freeze-dried overnight, then ground with 96% (v/v) ethanol and centrifuged. This was repeated with ethanol and then with ether. The residue was air-dried, then extracted at room temperature overnight with 8 vol. of M-KCl. Extraction with this salt solution was repeated twice more and the assay method of Bailey was continued from this point.

Trypsin treatment of actomyosin. This was done on the lines described by Szent-Györgyi (1953) for skeletal-muscle myosin. Actomyosin was used in the present work because experiments attempting to prepare free myosin from the uterus with Guba-Straub solution (see Szent-Györgyi, 1951) were unsuccessful. A solution of uterus actomyosin (containing about 10 mg. of protein/ml.) in 0·6 M-KCl was used. For every 10 ml., 1 ml. of 0·1 M-borate buffer (pH 8·8) and 0·5 mg. of crystalline trypsin were added. The digestion was carried on for 30 min. at 23°, then stopped by addition of 0·5 mg. of trypsin inhibitor dissolved in a small volume of 0·1 M-sodium acetate. In the first experiments the reaction was ended by adding 0·2 ml. of 0·1 M-DFP.

Ammonium sulphate fractionation. This was done according to Szent-Györgyi (1953), with slight modifications. The cooled digest was dialysed overnight at 4° against 10 vol. of 6·6 mM-potassium phosphate buffer (pH 7·0) and any precipitate was centrifuged off. The solution was brought to 0·4 satn. with $(NH_4)_2SO_4$ by adding the solid; the pH was maintained about 7·0 (as shown by tests with bromothymol blue on drops of the diluted solution) by addition of dilute ammonia. The precipitate (fraction 2) was removed by centrifuging. The content of $(NH_4)_2SO_4$ was raised to 0·55 satn. with ammonia addition if necessary, and this precipitate (fraction 3) was removed by centrifuging. Fractions 1–3 were each dissolved in a small volume of water and these solutions, as well as the supernatant (fraction 4) were dialysed against water until free from ammonia.

Measurement of adenosine triphosphatase activity. The reaction mixture (final volume 2 ml.) contained (unless otherwise stated): 2 mM-ATP; 0·066 M-glyoxaline buffer (pH 7·0); 0·01 M-$CaCl_2$. The concentration of KCl is given for the different experiments. The reaction was carried on for 3 min. at 28°. It was started by addition of enzyme, care being taken that the amounts used fell within the range of proportionality with the rate of reaction. The reaction was stopped by addition of 1 ml. of trichloroacetic acid (15%, w/v).

Measurement of viscosity. An Ostwald viscometer, needing 3·5 ml., was used; its flow time was 26·5 sec. for buffer at 22°.

Methods of estimation. Inorganic P was measured by the method of Fiske & Subbarow (1925) or by the method of Weil-Malherbe & Green (1951) if the quantities were below 20 μg. Protein N was estimated by the method of Chibnall, Rees & Williams (1943), followed by distillation in the apparatus of Markham (1942). For estimation of soluble N after trypsin treatment, the digest was brought to 2·5% (w/v) with trichloroacetic acid and kept at 4° overnight before the precipitated protein was centrifuged off (see Mihalyi, 1953).

Electrophoresis. This was done in the Tiselius apparatus (Perkin–Elmer Corp., Conn., U.S.A.; model 38) at 4°. The actomyosin solutions used contained 0·5 mg. of N/ml. in

0·4 M-KCl to which potassium phosphate (0·016 M; pH 6·75) had been added. They were dialysed overnight against this same buffered salt solution.

Sedimentation. For sedimentation of acto-H-meromyosin and of actin the Spinco ultracentrifuge model L was used, with cooling.

For the examination of the homogeneity of acto-H-meromyosin the model E analytical Spinco ultracentrifuge was used at 270 000 *g* and temp. 5–7°; the protein (1·1 mg. of N/ml.) was dissolved in 0·6 M-KCl containing 0·016 M-potassium phosphate (pH 7·0).

RESULTS

Tropomyosin in actomyosin preparations. The amount of tropomyosin present in an actomyosin preparation was estimated according to Bailey (1948). The thrice-precipitated actomyosin was made from 150 g. of non-pregnant pig uterus. After the heating procedure of the final stage of the assay, 16·5 mg. of protein was found in the supernatant. This amounts to about 2·5 % of the total protein of the actomyosin preparation. Another portion of the actomyosin preparation (also from 150 g. of uterus) was subjected to a leaching-out process, similar to that used by Perry (1953) with skeletal myofibrils. The actomyosin was kept for 12 days at 4° suspended in 0·039 M-borate buffer (pH 7·1) in the presence of a trace of toluene. At the end of this time the suspension was centrifuged at 4° at 10 000 *g* and the supernatant was used for stages I–III of Bailey's (1948) method of tropomyosin preparation. Protein was found to be 23 mg. (corresponding to about 3·5 % of the total protein). In each case the protein prepared showed the characteristic sharp fall in relative viscosity on addition of salt. Thus the first preparation, a solution containing 1·27 mg. of protein/ml. of 0·02 M-potassium phosphate buffer (pH 6·3), had a relative viscosity of 1·51, which fell to 1·14 on addition of KCl to 0·05 M.

Tropomyosin was also estimated in the whole myometrium from pregnant pig and compared with the actomyosin content. The procedure described by Needham & Cawkwell (1957) was used for the latter. The values found were 2·76 mg. of actomyosin N/g. wet wt. and 0·26 mg. of tropomyosin N. For bovine uterus Sheng & Tsao (1955) found about 0·25 mg. of tropomyosin N/g. wet wt., about 10 % of the structural proteins. The corresponding values for skeletal muscle are 10 mg. (Hasselbach & Schneider, 1950–51) and 0·8 mg. (Bailey, 1948).

Purification of uterus actomyosin. Repeated precipitation of the actomyosin by dilution to 0·25 M-KCl at pH 6·85 (see under Materials) resulted in the isolation of a protein which gave a single peak on electrophoresis. For the descending boundary $\mu = 3{\cdot}31–3{\cdot}36 \times 10^{-5}$ cm.^{2}/v/sec. After two precipitations at 0·04 M-KCl, three such precipitations were adequate in Expt. 1 (Table 1); in another experiment (Expt. 3, Table 1) in which a single precipitation at 0·25 M-KCl was carried out, electrophoresis showed a second small peak behind the main peak. In both cases the adenosine triphosphatase activity had been significantly raised by the purification. These experiments were done on rabbit myometrium.

Effects of trypsin-treatment on uterus actomyosin. Table 2 shows some of these experiments. In Expt. 3 the adenosine triphosphatase activity was increased by 60 % (tested in 0·5 M-KCl) and by nearly 500 % (tested in 0·1 M-KCl). As a result of these changes the adenosine triphosphatase activity was now about 40 % lower in the higher KCl concentration, as is found with skeletal-muscle actomyosin. That the enhanced ATP breakdown was due to true adenosine triphosphatase activity was shown by ascertaining that the reaction was complete when only one phosphate group was split off.

Fig. 2 shows the rapid fall in viscosity consequent on trypsin-treatment and the course of the formation of acid-soluble N during the trypsin-digestion. These curves may be compared with the rather

Table 1. *Effect of purification on the adenosine triphosphatase activity of actomyosin preparations*

Conditions of the activity tests were as described in the text.

Expt. no.	Procedure	Inorganic P liberated (μmoles/mg. of N/min.) With 0·1 M-KCl	With 0·5 M-KCl
1	Actomyosin made by two precipitations at 0·04 M-KCl	0·22	0·6
	With three further precipitations at 0·25 M-KCl	0·24	0·84
2	Actomyosin made by two precipitations at 0·04 M-KCl	0·08	0·59
	With one further precipitation at 0·25 M-KCl	0·12	0·74

Table 2. *Effect of treatment with trypsin and subsequent ammonium sulphate fractionation on actomyosin preparations*

In each case the uterus actomyosin had been precipitated three times at 0·04 M-KCl. The conditions of digestion and fractionation and the measurement of adenosine triphosphatase activity were as described in the text.

Expt. no.	Actomyosin preparation	Protein N (mg.)	Inorganic P liberated (μmoles/mg. of N/min.) With 0·1 M-KCl	With 0·5 M-KCl
3	From pig uterus (not pregnant)			
	Digest before trypsin-treatment	30	0·3	0·74
	Digest after trypsin-treatment	—	1·7	1·2
	Fraction 1	2·61	0·15	0·1
	Fraction 2	7·7	4·5	2·0
	Fraction 3	3·7	No activity	—
4	From pregnant rabbit uterus			
	Digest before trypsin-treatment	48	0·21	0·52
	Digest after trypsin-treatment	—	1·5	0·89
	Fraction 1	2·7	0·23	0·15
	Fraction 2	17·3	2·2	1·6
	Fraction 3	4·8	No activity	—
	Fraction 4	4·0	No activity	—
5	From pregnant pig uterus			
	Digest before trypsin-treatment	51	0·12	0·34
	Digest after trypsin-treatment	—	1·08	0·91
	Fraction 2	14·3	2·6	2·1
6	From pregnant rabbit uterus			
	Digest before trypsin-treatment	44·4	0·15	0·42
	Digest after trypsin-treatment	—	0·79	0·59
	Fraction 1	2·52	1·54	0·99
	Fraction 2	12·5	3·27	1·9
	Fraction 3	3·6	No activity	—
	Fraction 4	3·2	No activity	—
	From rabbit skeletal muscle; once pptd. at 0·04 M-KCl, once at 0·3 M-KCl			
	Digest before trypsin-treatment	47·4	4·5	1·23
	Digest after trypsin-treatment	—	5·3	1·4
	Fraction 1	3·0	2·0	1·2
	Fraction 2	11·8	8·2	4·1
	Fraction 3	11·3	11·6	6·8
	Fraction 4	—	No activity	—

similar ones for skeletal-muscle myosin, given by Gergely (1953) for viscosity changes, and by Mihalyi & Szent-Györgyi (1953*b*) and Gergely *et al.* (1955) for acid-soluble N.

Attempts were then made, by application of the $(NH_4)_2SO_4$ fractionation method of Szent-Györgyi (1953), to see whether a protein fraction corresponding to the acto-H-meromyosin from skeletal-muscle myosin, had been formed. As for skeletal-muscle protein this method has only been described for separation of the products of myosin digestion; in Expt. 6 (Table 2) a digestion of skeletal-muscle actomyosin was included.

A study was made of the time course of the increase in adenosine triphosphatase activity during the treatment with trypsin. Small samples were removed at different time intervals, treated at once with trypsin inhibitor and tested for adenosine triphosphatase activity. The results are shown in Fig. 3.

This fractionation with actomyosin as the substrate for digestion did not give the clear-cut results obtainable with skeletal-muscle myosin. From the adenosine triphosphatase results it appears that acto-H-meromyosin contaminated fraction 1 and could spread over fractions 2 and 3. Also recovery of the protein was incomplete, since solution of the fractions and complete clearing of the supernatants on centrifuging were often very slow. About 66% of the original protein was usually accounted for; more than 80% of the adenosine triphosphatase activity could be recovered in the fractions.

Another method for the separation of the acto-H-meromyosin from the uterus was therefore tried, based on a procedure described by Mihalyi & Szent-Györgyi (1953*b*) for separating acto-H-meromyosin after actin had been added to digested myosin.

A preparation was made from pregnant pig uterus (150 g.) by the procedure for obtaining actomyosin electrophoretically homogeneous. A

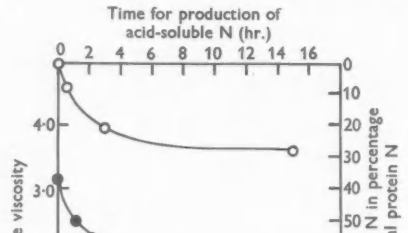
Time for production of acid-soluble N (hr.)
0 2 4 6 8 10 12 14 16
Relative viscosity
4·0
3·0
2·0
1·0
0
Acid-soluble N in percentage of original protein N
0 10 20 30 40 50 60 70 80 90
0 10 20 30 40 50 60 70 80
Time for viscosity fall (min.)

Fig. 2. Effect of trypsin-treatment on the relative viscosity of pig-uterus actomyosin, and on formation of acid-soluble N from rabbit-uterus actomyosin. Viscosity measurements were made at 22° in an Ostwald viscometer. For protein precipitation 2·5% of trichloroacetic acid was added and the mixtures were kept 15 hr. at 4° before centrifuging. ●, Viscosity change; ○, acid-soluble N as percentage of the original protein N.

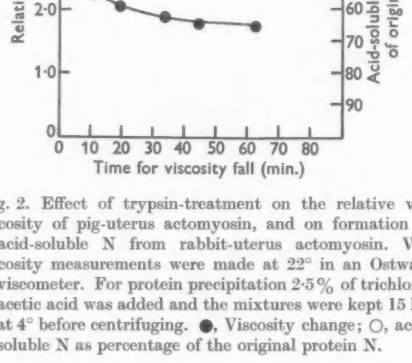

Fig. 3. Effect of trypsin-treatment on the adenosine triphosphatase activity of pig-uterus actomyosin, tested in 0·1 M-KCl (●) and in 0·5 M-KCl (○). The digests were prepared as described in the text, and the trypsin activity was stopped in each sample by addition of trypsin inhibitor.

solution (23 ml. containing 7·5 mg. of protein/ml. in 0·6 M-KCl) was treated as usual with trypsin. After addition of trypsin inhibitor the solution was diluted with 0·6 M-KCl to a content of 3·7 mg. of protein/ml. and centrifuged for 2 hr. at 100 000 *g*. The semi-transparent pellets deposited contained 49% of the original protein. In the supernatant 45% was recovered.

The pellets were dissolved in 0·5 M-KCl buffered with 0·01 M-potassium phosphate (pH 7·0). A portion was used for the formation of H-meromyosin by dissociating the acto-H-meromyosin. A solution containing 10 mg. of protein/ml., 3·3 mM-ATP and 5 mM-$MgCl_2$ was centrifuged for 2 hr. at 100 000 *g*. A small pellet, presumably of actin and undissociated acto-H-meromyosin, was deposited. The second supernatant (H-meromyosin) as well as the first supernatant and the solution of the first pellets (acto-H-meromyosin) were tested for adenosine triphosphatase activity (Table 3).

In the analytical ultracentrifuge the acto-H-meromyosin showed one main component, S_{20} about 73 (compare Mihalyi & Szent-Györgyi, 1953*b*). No protein resembling L-meromyosin was precipitated from the first supernatant on prolonged dialysis against 0·04 M-KCl containing 3 mM-phosphate, pH 7·0 (compare Velick, 1956).

The effect of the same treatment with trypsin on the adenosine triphosphatase activity of the particulate fraction from the myometrium was also tested. There was a marked clearing of the suspension and about 20% of the protein N became acid-soluble, but the adenosine triphosphatase activity did not increase by more than 10%.

Possible causes of the low adenosine triphosphatase activity of purified uterus actomyosin. Since the results of trypsin-treatment show that the uterus actomyosin has potentially a high adenosine triphosphatase activity, an investigation was made of possible causes of partial inactivation during the isolation and purification of the protein, and of possible reasons for the specially low activity in 0·1 M-KCl.

Table 3. *Adenosine triphosphatase activity of the products of trypsin-digestion of uterus actomyosin*

Conditions of trypsin-digestion and measurement of adenosine triphosphatase activity were as described in the text.

	Inorganic P liberated (μmoles/mg. of N/min.)
Digest centrifuged (2 hr. at 100 000 *g*)	
Supernatant	0·38
Pellet	5·5
Solution of pellet in 0·5 M-KCl (containing 0·01 M-potassium phosphate, pH 7, 3·3 mM-ATP and 5 mM-$MgCl_2$) centrifuged (2 hr. at 100 000 *g*)	
Supernatant	4·6

The effect of –SH reagents was tried, but glutathione (0·01 M) and BAL (5 mM–75 μM) were without effect. Besides its effect in restoration of –SH groups, BAL has marked capacity for combination with heavy metals (Webb & van Heyningen, 1947). With the idea of removing these possible inhibitory factors, glycine (0·04 M) was also used but the enzyme activity was unchanged. The glyoxaline buffer used in the routine tests probably also protected the enzyme against effects of heavy metals.

The possibility that combination with actin might be masking active groups was tested by using much higher ATP concentration (8 mM) in the hope of increasing dissociation of the actomyosin. Little effect was shown when the test was made in 0·5 M-KCl; in 0·1 M-KCl there was even less activity with the high substrate concentration.

The suggestion was made that one effect of trypsin might be to remove an inhibitory substance bound to the actomyosin. This was investigated in the following way. Trypsin-treatment was carried out as usual and, after dilution to 4 mg. of protein/ml., the digest was centrifuged for 2 hr. at 100 000 *g*. The pellet of acto-H-meromyosin was dissolved in 0·6 M-KCl and centrifuged again, to remove any remaining trypsin. The second pellet was dissolved in 0·6 M-KCl. In order to find whether any diminution of the high adenosine triphosphatase activity occurred upon mixing with untreated actomyosin, a solution in 0·5 M-KCl containing 0·12 mg. of acto-H-meromyosin N/ml. and 1·4 mg. of actomyosin N/ml. was left at 4° for 12 hr. The adenosine triphosphatase activity of the diluted mixture was compared with the sum of the activities of the actomyosin and acto-H-meromyosin (Table 4). It is seen that there is no diminution in activity but an increase, which could be perhaps accounted for by adsorption of a trace of trypsin. The activation is slight; thus if it concerns only the actomyosin activity it can be calculated that the P liberated has risen from 0·12 to 0·15 μmole/mg. of N/min. after standing with the acto-H-meromyosin. This experiment gives no evidence for the presence of an inhibitory factor, but the latter is not ruled out since it might not affect the acto-H-meromyosin under the conditions used.

Adenosine triphosphatase activity of native myofilaments. It is possible that the procedure of solution of the actomyosin and reprecipitation might themselves affect the original adenosine triphosphatase activity; such changes have been reported by Perry (1951) for skeletal myofibrils. Efforts were therefore made to get preparations of native myofilaments free from cell particles and collagen fibres. This aim has not yet been satisfactorily achieved, but since some conclusions can be drawn from the experiments they will be described here.

Table 4. *Investigation of the possible effect of actomyosin on the adenosine triphosphatase activity of acto-H-meromyosin*

Conditions of the activity tests were as described in the text.

	Inorganic P liberated in 5 min. at 28° (μg.)	
	With 0·1 M-KCl	With 0·5 M-KCl
Actomyosin N (0·07 mg.)	1·2	5·0
Acto-H-meromyosin N (0·019 mg.)	6·2	5·9
Actomyosin (0·07 mg.) plus 0·0067 mg. of acto-H-meromyosin N:		
Observed	4·0	9·1
Calculated for the mixture	3·4	7·1

When the collagenase treatment had been given in the preparation of the homogenate (see Methods section and Fig. 1), after the second homogenization and centrifuging the main residue R was covered by a fine sludge which could easily be poured or pipetted off separately (R_1). R_3 was obtained in the same way after the third homogenization and centrifuging. When no collagenase treatment was given, this layer was absent and only R_2 and R_4 were obtained. The yields were very poor and variable: only about 50 mg. of protein N/100 g. wet wt. for R_1 plus R_3, and much less for R_2 plus R_4. Treated with van Gieson's stain and examined microscopically, all four fractions showed much muscle and little connective tissue. No unbroken cells and usually no nuclei, or very few, were seen. Under the phase-contrast microscope at magnification × 850, masses of fine fibrils, rather more than 50 mμ in diameter, were seen lying side by side or fanning out. Since the individual filaments in the uterus-muscle cell examined with the electron microscope were found to be only about 50 Å in width (Shoenberg, 1958) the fibrils seen here must consist of artificially aggregated filaments.

Representative analyses of these filaments are given in Table 5. The protein going into solution upon treatment with 0·5 M-KCl (containing 0·05 M-potassium phosphate, pH 7, and 1·5 mM-ATP) was taken as actomyosin, the assumption being made that soluble protein from the cell particles would already have been extensively extracted in the initial procedures with solutions of low ionic strength (compare Hillarp & Nilson, 1954). The undissolved residue was treated with 0·1 N-NaOH at 4° for 20 hr.; the protein soluble in NaOH was taken as particle protein, that insoluble as collagen.

Table 5 shows that preparations could be obtained consisting of muscle probably to the extent of 60–70%; the collagen content was always low

Table 5. *Nitrogen distribution in the fractions obtained by centrifuging uterus homogenates*

Preparation of the fractions R_1, R_3 and R_4 and the method of analysis are described in the text.

Expt. no.	Fraction used	Conditions	Total N	Actomyosin N	Particle N	Collagen N
F3	R_4	No collagenase treatment. The 0·5M-KCl soln. was centrifuged (1500 *g* for 20 min.) to obtain the particles	6·86	4·9	1·68	0·19
F6	R_1+R_3	Collagenase treatment for 20 hr. The 0·5M-KCl soln. was centrifuged (1500 *g*) to obtain the particles	7·0	4·5	0·6	0·08
		The 0·5M-KCl ('actomyosin') solution was centrifuged again (15000 *g* for 40 min.)	—	3·6	0·63	—

Table 6. *Assessment of the adenosine triphosphatase activity of the native myofilaments of the uterus*

Preparation of the fractions R_1–R_4 and the method of subdividing these into actomyosin solution and particles + collagen are described in the text. Adenosine triphosphatase activity is expressed as μmoles of inorganic P liberated/mg. of N/min. estimated in 0·1M-KCl. In Expts. F2, F6 and F10, pregnant rabbit uterus was used; in F3 and F9, pregnant pig uterus was used. In Expts. F2 and F3, the actomyosin solution was centrifuged for 20 min. at 1500 *g* to get the particles + collagen. In Expts. F6, F9 and F10, centrifuging at this stage was for 60 min. at 15 000 *g*.

Expt. no.	Fraction used	Actomyosin (%)	Particles + collagen (%)	Adenosine triphosphatase activity of R fractions	Adenosine triphosphatase activity of particles + collagen	Particle contribution to adenosine triphosphatase activity in 1 mg. of R-fraction N	Myofilament contribution to adenosine triphosphatase activity in 1 mg. of R-fraction N	Adenosine triphosphatase activity of myofilaments
F2*	R_2+R_4	56	44	0·96	1·6	0·69	0·27	0·5
F3*	R_4	72	28	0·6	0·8	0·22	0·38	0·52
F6†	R_1+R_3	70	30	2·0	2·2	0·66	1·34	1·9
F9*	R_2+R_4	38	62	0·7	0·71	0·45	0·35	0·6
F10‡	R_1+R_3	63	37	0·55	0·53	0·2	0·35	0·6
	R_2+R_4	45	55	1·2	1·7	0·94	0·26	0·6

* No collagenase treatment. † Collagenase treatment for 20 hr. ‡ Collagenase treatment for 4 hr.

Table 7. *Effect of collagenase treatment on the adenosine triphosphatase activity of actomyosin gels from the uterus*

Adenosine triphosphatase activity was measured as described in the text.

Expt. no.	Conditions	Inorganic P liberated (μmole/mg. of N/min.) In 0·1 M-KCl	In 0·5 M-KCl
1	In 0·04 M-borate buffer, pH 7·0; 12 hr. at 4°		
	Control	0·098	0·49
	With collagenase 12 hr.	0·74	0·89
	With collagenase 18 hr.	0·69 0·86	0·74 0·94
2	In 0·04 M-borate buffer, pH 7·25; 15 hr. at 4°		
	Control	0·36	0·86
	With collagenase	1·00	1·29

but in some cases the particle content was higher than is shown here. With the very finely divided R_2 and R_4 it was assumed that all the particles which had come down originally with the fibrils would come down at this centrifugal force (1500 *g*) from the KCl solution. With R_1 and R_3, less finely divided, it was found that greater centrifugal force was needed to remove all the particles, presumably because some lighter ones had been carried down entangled in the interstices of the fibrils. This would not be surprising when one remembers the extremely intimate relations of the mitochondria and cytoplasmic reticulum with the myofilaments, as seen in electron micrographs of the uterus-muscle cell (Shoenberg, 1958). In later experiments the KCl solution was always centrifuged at 15 000 *g* to obtain the particle-collagen fraction.

A summary of the results on the adenosine triphosphatase activity of the R_1–R_4 fractions and the particle-collagen fractions is given in Table 6. It can be seen that the calculated adenosine triphosphatase activity of the myofibrillar fractions amounted to about 0·6 μmole of P/mg. of N/min. when tested in 0·1 M-KCl, an activity about equal to that of purified actomyosin preparations tested in 0·5 M-KCl. It thus seems that the very low adenosine triphosphatase activity of purified preparations tested in 0·1 M-KCl may be caused by the repeated solution and reprecipitation of the protein. The adenosine triphosphatase activity of the particles was always found to be higher in the rabbit than in the pig uterus, as observed previously by Needham & Cawkwell (1956); the very low value found in Expt. F10 (Table 6) for the particle-containing fraction from R_1 plus R_3 is probably to be explained by the very high nucleic acid content noticed in this particular case. Nuclei had been in fact observed in the microscopic examination of R_1.

The high value for myofibrillar adenosine triphosphatase in Expt. F6 (with collagenase treatment) must be mentioned. This observation led to the examination of the effect of collagenase treatment on purified actomyosin gels. Thrice-precipitated actomyosin was used as a suspension in 0·04 M-borate buffer and left at 4° for different lengths of time, with collagenase (0·18 mg./ml.). The gels were then centrifuged, washed to remove collagenase, dissolved in 0·5 M-KCl and tested for adenosine triphosphatase activity. Table 7 shows the results: an increase in activity, particularly in the activity measured at low ionic strength. In another experiment the collagenase treatment was done at room temperature and the resulting viscosity change was followed. This was completed in 45 min. and was rather less than the viscosity change found with trypsin. During this time no change was found in adenosine triphosphatase activity. As a result of this observation, in Expt. F10 (Table 6) collagenase treatment was carried out at 4° for only 4 hr., with the aim of getting some effect on the collagen without effect on the myofibrillar adenosine triphosphatase. This seems to have been accomplished, since the adenosine triphosphatase activity resembled that in experiments without collagenase, and a good yield of R_1 and R_3 was obtained.

The adenosine triphosphatase activity of the actomyosin solutions made by extracting fractions R_1–R_4 with 0·5 M-KCl was tested in some experiments. It was always lower than the calculated myofibrillar adenosine triphosphatase; the difference between activities in 0·1 M-KCl and 0·5 M-KCl was often much less than with purified actomyosin.

DISCUSSION

Our results do not confirm the finding of Snellman & Tenow (1954) that by dilution of uterus-muscle extract (made with 0·5 M-potassium chloride containing 0·1 M-potassium phosphate, pH 7·0) to 0·1 M-potassium chloride, a protein complex is

obtained containing roughly 40% of actin and 20–25% of tropomyosin. As has been described here, not more than 3·5% of tropomyosin could be found in the dilution precipitates even without extensive purification. The precipitates of Snellman & Tenow (1954) also contained nucleic acid and they suggest that the tropomyosin was present as nucleotropomyosin. It is true that nucleotropomyosin can be obtained from uterus muscle (Sheng & Tsao, 1955, from the cow; D. M. Needham & J. M. Williams, unpublished work, from the pig and rabbit, by the method of Sheng & Tsao). However, it is likely (compare Hamoir, 1952) that the nucleotropomyosin (if not an artifact but actually occurring as such in the tissue) is dissociated on the treatment with organic solvents involved in Bailey's method for tropomyosin isolation. So both nucleotropomyosin and tropomyosin would be estimated in the actomyosin precipitates by the methods used here. Sheng & Tsao (1955) found that the yield of tropomyosin itself was the same, whether the muscle was treated by Bailey's method (giving free tropomyosin) or by their own method, giving mainly nucleotropomyosin. Perry & Corsi (1958) found that by a still milder method of extraction of myofibrils a higher amount of tropomyosin could be obtained (10–12% of the total N of the myofibrils). However, it seems that even if up to half the tropomyosin were destroyed or left unextracted in the method of Bailey or of Sheng & Tsao, not more than about 7% of our dilution precipitates could have consisted of tropomyosin. Kominz, Saad, Gladner & Laki (1957) remark that tropomyosin impurity in myosin B (myosin plus actomyosin) preparations from human uterus becomes more significant if ATP is omitted from the extraction medium.

The mechanism of the trypsin effect in raising the adenosine triphosphatase activity of uterus actomyosin remains obscure. The fall in viscosity roughly parallels the increase in adenosine triphosphatase activity in 0·5M-potassium chloride. With skeletal-muscle myosin Mihalyi & Szent-Györgyi (1953*a*) found the viscosity fall to parallel the appearance of the H- and L-meromyosin assessed from sedimentation patterns. From Fig. 3 it is seen that the increase in activity measurable in 0·5M-potassium chloride is complete in about 10 min., whereas the increase measurable in 0·1M-potassium chloride needs about 30 min. This suggests that, besides splitting the protein into the meromyosins, trypsin may also affect the adenosine triphosphatase activity of the acto-H-meromyosin formed.

The effects of collagenase are difficult to interpret, since the preparation is described as containing traces of proteinase and peptidase; it is thus possible that the effects on actomyosin are due, in part at least, to an unspecific proteinase. Chymotrypsin (Gergely *et al.* 1955; Middlebrook, 1958) and subtilisin (Middlebrook, 1958) both act upon myosin to give very similar products to those given by trypsin, and Middlebrook suggests that the reaction is non-hydrolytic. The effect of short collagenase treatment in causing fall in viscosity in uterus actomyosin without increase in adenosine triphosphatase activity differed from that of trypsin, with which fall in viscosity and increase in adenosine triphosphatase activity both started immediately. Collagenase treatment had no effect on Ca^{2+} ion-activated activity of adenosine triphosphatase in skeletal myofibrils (Perry, 1951).

The acto-H-meromyosin prepared from uterus is similar to that from skeletal muscle in sedimentation rate and solubility. Its adenosine triphosphatase activity is of the same order, somewhat less in Expt. 6 (Table 2), but in Table 3 higher than that given by Mihalyi & Szent-Györgyi (1953*b*) in their description of acto-H-meromyosin made from skeletal-muscle actomyosin by a similar sedimentation procedure.

So far no preparation of L-meromyosin has been obtained from uterus actomyosin. Further work is needed here.

The evidence from this work suggests that the myofilaments of pregnant-uterus muscle have indeed a low adenosine triphosphatase activity (about 10% of that of skeletal myofibrils); this value agrees with that found in 0·5M-potassium chloride solutions for actomyosin preparations from the uterus. Since the myofilament preparations showed similar activities in 0·5 and 0·1M-potassium chloride, it seems that the very low activity of uterus actomyosin preparations tested in 0·1M-potassium chloride (that is, in the gel form) may be an artifact.

The low adenosine triphosphatase activity of the myofilament preparations investigated at various stages of pregnancy is puzzling when one considers the task of uterus muscle at parturition. It may be of interest to consider the possible significance of the finding that from uterus actomyosin acto-H-meromyosin can be obtained, in amount comparable with that obtained from skeletal-muscle actomyosin and of similar activity. Whether an unmasking of this potential adenosine triphosphatase activity occurs *in vivo* at the end of pregnancy is not yet known. If so, some unmasking agent which did not, like trypsin, simultaneously disrupt myosin would presumably be at work.

SUMMARY

1. Actomyosin electrophoretically homogeneous has been prepared from pregnant rabbit uterus.

2. Short treatment of uterus actomyosin with trypsin led to increased adenosine triphosphatase

activity of the digest: 400–800% when tested in 0·1M-potassium chloride, about 50% when tested in 0·5M-potassium chloride. By ultracentrifuging, a product could be separated from the digest comprising half the total protein and containing the whole adenosine triphosphatase activity. This product, in its high adenosine triphosphatase activity (about 5 μmoles of phosphorus liberated/mg. of nitrogen/min.), sedimentation rate and solubility resembled the acto-H-meromyosin obtained by similar treatment of skeletal-muscle actomyosin.

3. No evidence could be obtained that the very low adenosine triphosphatase activity (especially when tested in 0·1M-potassium chloride) of purified actomyosin from uterus was due to oxidation of –SH groups or to inhibition by heavy metals.

4. Preparations containing myofilaments were made from the uterus by homogenization in a medium of low ionic strength followed by differential centrifuging. The adenosine triphosphatase activity of these myofilaments (about 0·5 μmole of phosphorus liberated/mg. of nitrogen/min.) was the same tested in 0·1 and 0·5M-potassium chloride and resembled that of purified preparations tested in 0·5M-potassium chloride.

5. Treatment of uterus homogenates with a collagenase preparation made it possible to obtain better yields of the myofilament-containing preparation; these showed enhanced adenosine triphosphatase activity, probably due to a direct effect of the collagenase preparation on the myofilaments.

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REFERENCES

Bailey, K. (1948). *Biochem. J.* **43**, 271.
Britton, H. T. S. (1955). In *Hydrogen Ions*, 4th ed., vol. 1, p. 361. London: Chapman and Hall Ltd.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Gergely, J. (1953). *J. biol. Chem.* **200**, 543.
Gergely, J., Gouvea, M. A. & Karibian, D. (1955). *J. biol. Chem.* **212**, 165.
Hamoir, G. (1952). *Biochem. J.* **50**, 140.
Hasselbach, W. (1952). *Z. Naturf.* B, **7**, 163.
Hasselbach, W. & Schneider, G. (1950–51). *Biochem. Z.* **321**, 462.
Hillarp, N.-Å. & Nilson, B. (1954). *Acta physiol. scand.* **31**, Suppl. 113, p. 79.
Kominz, D. R., Saad, F., Gladner, J. A. & Laki, K. (1957). *Arch. Biochem. Biophys.* **70**, 16.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Middlebrook, W. R. (1958). *Abstr. 4th int. Congr. Biochem. Vienna*, p. 84.
Mihalyi, E. (1953). *J. biol. Chem.* **201**, 197.
Mihalyi, E. & Szent-Györgyi, A. G. (1953*a*). *J. biol. Chem.* **201**, 189.
Mihalyi, E. & Szent-Györgyi, A. G. (1953*b*). *J. biol. Chem.* **201**, 211.
Needham, D. M. & Cawkwell, J. M. (1956). *Biochem. J.* **63**, 357.
Needham, D. M. & Cawkwell, J. M. (1957). *Biochem. J.* **65**, 540.
Needham, D. M. & Cawkwell, J. M. (1958). *Biochem. J.* **68**, 31P.
Palitzsch, S. (1915). *Biochem. Z.* **70**, 333.
Perry, S. V. (1951). *Biochem. J.* **48**, 257.
Perry, S. V. (1953). *Biochem. J.* **55**, 114.
Perry, S. V. & Corsi, A. (1958). *Biochem. J.* **68**, 5.
Perry, S. V. & Grey, T. C. (1956). *Biochem. J.* **64**, 184.
Sheng, P.-K. & Tsao, T.-C. (1955). *Sci. Sinica*, **4**, 157.
Shoenberg, C. F. (1958). *Biophys. Biochem. Cytol.* **4**, 609.
Snellman, O. & Tenow, M. (1954). *Biochim. biophys. Acta*, **13**, 199.
Szent-Györgyi, A. (1951). *Chemistry of Muscular Contraction*, 2nd ed., p. 146. New York: Academic Press Inc.
Szent-Györgyi, A. G. (1953). *Arch. Biochem. Biophys.* **42**, 305.
Velick, S. F. (1956). *Biochim. biophys. Acta*, **20**, 228.
Webb, E. C. & van Heyningen, R. (1947). *Biochem. J.* **41**, 74.
Weil-Malherbe, H. & Green, R. H. (1951). *Biochem. J.* **49**, 286.

The Chemistry of Xanthine Oxidase

4. THE PROBLEMS OF ENZYME INACTIVATION AND STABILIZATION*

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It was stated in previous papers (Avis, Bergel & Bray, 1955; Avis, Bergel, Bray, James & Shooter, 1956; Avis, Bergel & Bray, 1956) that the enzymic activity of purified xanthine oxidase from cow's milk, like that of other enzymes, deteriorates in aqueous solution under a variety of mild conditions. Since then we recorded in a preliminary communication (Bergel & Bray, 1956) the stabilizing effects of sodium salicylate on this activity and mentioned briefly during a symposium the inactivation of the enzyme by certain metals (Bergel & Bray, 1958).

We have now extended our studies of loss and preservation of the catalytic power of xanthine oxidase in order to establish optimum conditions for storage and utilization of the enzyme in biological experiments (cf. Bergel, Bray, Haddow & Lewin, 1957; Haddow, de Lamirande, Bergel, Bray & Gilbert, 1958; Bergel, 1959). These studies should also help in elucidating more rapidly the chemical nature of the enzyme and in its separation from various closely related inactive compounds.

In this paper we demonstrate that at least three inactivation mechanisms, namely interaction with metals, photo-oxidation and gross denaturation, are operative, and that some of the losses of activity can be counteracted by a number of chemicals.

EXPERIMENTAL

Materials

Xanthine oxidase solutions. Samples of xanthine oxidase, prepared from seven different batches of buttermilk, were used; some were prepared by the method of Avis *et al.* (1955), the purification being taken to stage M 5, M 6 or M 7, while others were prepared by ammonium sulphate treatment (cf. Haddow *et al.* 1958). The samples had E_{280}/E_{450} ratios ranging from 5·4 to 10·7 and activity/E_{450} ratios of from 20 to 90. The ratios E_{280}/E_{450} and activity/E_{450} are referred to as 'PFR' and 'AFR' in previous publications (Avis *et al.* 1955). However, most of the experiments, apart from the work on activation by ethylenediaminetetra-acetate (EDTA) (applicable to one enzyme preparation only), and those concerning photo-oxidation at 37°, were carried out on more than one sample with very similar results.

* Part 3: Avis, Bergel & Bray (1956).

Chemicals. These were generally of A.R. or laboratory-reagent grade. Water was glass-distilled and then passed down a deionizing column. Phosphate and acetate buffers were prepared according to Green (1933), 2-amino-2-hydroxymethylpropane-1:3-diol–maleic acid (tris–maleate) buffer was prepared according to Gomori (1955), and pyrophosphate buffer (pH 8) according to Avis *et al.* (1955). Crystallized bovine-plasma albumin was obtained from Armour Laboratories Ltd., Eastbourne, Sussex, 'Tween 80' (T.B. culture grade) from Honeywill and Stein Ltd., London, and reduced diphosphopyridine nucleotide (DPNH) and sodium *p*-chloromercuribenzoate (*p*CMB) from Sigma Chemical Co., U.S.A. 1:4-Naphthaquinol was prepared by reducing the quinone with zinc dust and dilute hydrochloric acid, filtering hot and leaving to crystallize; it had m.p. 175–182°. Peracetic acid was prepared by the method of Greenspan (1946).

Methods

Enzyme assays. The activity of xanthine oxidase solutions was generally measured by following uric acid formation spectrophotometrically at 295 mμ with xanthine as substrate and oxygen as electron acceptor. The conditions used (0·05 M-pyrophosphate buffer, pH 8·2; approx. 0·1 mM-xanthine; 24°) were those of Avis *et al.* (1955). The solutions from the stability experiments were usually diluted about 100-fold in the assays. The diaphorase-like activity of xanthine oxidase (Table 5) was also measured spectrophotometrically, by following the disappearance of DPNH at 340 mμ with ferricyanide as electron acceptor, under conditions recommended by V. Massey (personal communication), namely 0·2 M-acetate buffer, pH 4·6 and 24°. DPNH (final concn. 0·17 mM) was added immediately before the enzyme with 1·2 mM-potassium ferricyanide. Significant reaction occurred without enzyme, and corrections were made for the 'blank' rate.

Stability experiments. Samples of the xanthine oxidase solutions were diluted with the buffer or other reagents and stored for the appropriate time in a bath at the temperatures indicated. The solutions generally had volumes of about 0·2 ml. in 5 mm. × 50 mm. test tubes with rubber caps. The concn. of xanthine oxidase in the solutions was calculated from E_{450} (cf. Avis, Bergel & Bray, 1956). Xanthine oxidase activity was measured on samples taken immediately after making up the mixtures and then on further samples, after appropriate periods of incubation, and results were generally expressed as a percentage of the activity found immediately after mixing. For experiments on copper inactivation in the cold, the $CuSO_4$ was

added last, all solutions and pipettes being pre-cooled. For the experiment under nitrogen reported in Fig. 8*a* and *b*, the enzyme solution was stored in a shallow layer under the gas for 24 hr. in the cold, then filled into glass capillaries (melting-point tubes) in a glove cabinet filled with nitrogen. The tubes were sealed at each end inside the box with putty (Belco cellulose putty, I.C.I. Ltd.), then placed in a blackened test-tube immersed in the water bath. One capillary was removed and opened for the initial and each subsequent assay.

Theory of stabilization by a competitive inhibitor. A competitive inhibitor might be expected to protect an enzyme by combining with the active centre to form a complex whose molecules decompose at a lower rate than do those of the free enzyme. If certain simplifying assumptions are made this effect can readily be calculated, and the theoretical curve in Fig. 9 for xanthine oxidase stabilization by salicylate was drawn from the equation given below, the values of the constants being obtained by trial and error. To obtain the equation it is assumed that the usual Michaelis conditions apply (i.e. the enzyme concentration is low) and that for both, the combined and free enzyme, the proportion which remains unchanged at the end of a period of heating is independent of their initial concentrations. It can then be shown that

$$A_c = A_\infty\left[\frac{1}{1+K/C}\right]+A_0\left[1-\frac{1}{1+K/C}\right],$$

where C is the concentration of the inhibitor and K is the dissociation constant of the complex that it forms with the enzyme. A is the fraction of the enzyme activity which remains at the end of the heating period. (This is measured after considerable dilution of the enzyme so that competitive inhibition does not interfere with the activity measurements.) The subscripts $_0$, $_c$ and $_\infty$ refer respectively to experiments carried out with the inhibitor at zero concentration, at concentration C and at a concentration sufficiently high to 'saturate' the enzyme.

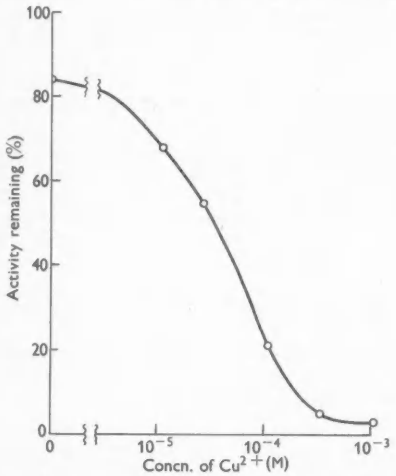

Fig. 1. Effect of various concentrations of cupric sulphate on xanthine oxidase. Samples were heated for 2·5 hr. at 37° in mM-acetate buffer, pH 5; activities are expressed as a percentage of that in an unheated sample without copper; the concentration of xanthine oxidase was 0·1%.

RESULTS

Inactivation by copper and other metal-containing reagents

We have reported before on inactivation of xanthine oxidase by copper, mercury, lead and silver salts (Bergel & Bray, 1958).

Philpot (1938) had already studied the effect of Cu^{2+} ions on xanthine oxidase, and an experiment carried out by us confirmed the sensitivity of our enzyme preparations to this metal (Fig. 1). As with hydrogen peroxide treatment and photo-oxidation at 37°, as described below, loss of enzymic

Table 1. *Loss of activity of xanthine oxidase in the presence of* Cu^{2+} *ions*

The solutions were incubated for 24 hr. at 0° with 0·1% xanthine oxidase and 0·1 mM-$CuSO_4$.

pH	Buffer (M)				EDTA (mM)	Activity remaining (%)
	Acetate	Tris–maleate	Pyrophosphate	Phosphate		
5·0	0·1	—	—	—	—	98
5·0	0·1	—	—	—	10	143
5·0	—	0·1	—	—	—	108
5·0	—	0·1	—	—	10	103
4·8	—	—	0·1	—	—	107
6·1	—	0·1	—	—	—	100
5·9	—	—	0·1	—	—	88
5·8	—	—	—	0·8	—	27
6·2	—	—	—	0·1	—	0
6·2	—	—	—	0·1	10	95
6·9	—	0·1	—	—	—	22
6·9	—	0·1	—	—	10	102
7·4	—	—	0·1	—	—	97
7·3	—	—	—	0·1	—	0
7·3	—	—	—	0·1	10	97
8·3	—	0·1	—	—	—	99
8·1	—	—	0·1	—	—	99

function in presence of copper took place without any visible change in the appearance of the clear reddish brown xanthine oxidase solutions, but obviously the activity/E_{450} ratio decreased. The reaction was found to be very sensitive to pH and to the nature and concentration of the buffers. This is illustrated by data shown in Table 1. Inactivation occurred only in the region of pH 6–7 at which denaturation at 56° was at a minimum (cf. Fig. 10). Pyrophosphate buffer or EDTA afforded virtually complete protection under all conditions tested, while with phosphate buffer the stability was greater at a high buffer concentration than at a low one. With the particular sample of xanthine oxidase used for these experiments (activity/E_{450} 20, E_{280}/E_{450} 7·4), EDTA in acetate buffer, at pH 5, produced a significant increase of activity over and above the activity of the sample before addition of the metal. This result was reproducible, but could not be observed with other samples of xanthine oxidase. The explanation for this may be that this enzyme concentrate had accidentally become contaminated with copper during dialysis against water with a high conductivity. That EDTA under suitable conditions reversed inactivation by added Cu^{2+} ions is shown in Fig. 2.

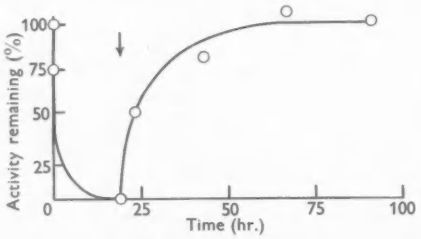

Fig. 2. Reversal of Cu^{2+} inactivation by EDTA. Xanthine oxidase (0·1%) in 0·1 M-phosphate buffer, pH 6·2, was treated at 0° with 0·1 mM-$CuSO_4$ at zero time. The first point represents the activity before adding Cu^{2+} and the second that immediately after the addition. After 19 hr., the sample was diluted by the addition of an equal volume of acetate buffer containing EDTA (final concentration 0·5 and 0·01 M respectively). The final pH was 5·0–5·2.

The protective effects of EDTA, sodium salicylate and sodium benzoate against copper inactivation at 5° and pH 7 are shown in Fig. 3: while salicylate and, to a lesser degree, benzoate reduced the rate of inactivation, EDTA prevented it completely. Salicylate, even at 37°, as shown in Table 2, could protect the enzyme completely against the effects of lower concentrations of copper.

Ascorbic acid at pH 7 markedly increased the rate of inactivation by copper, as can be seen in Table 3. EDTA cancelled this effect.

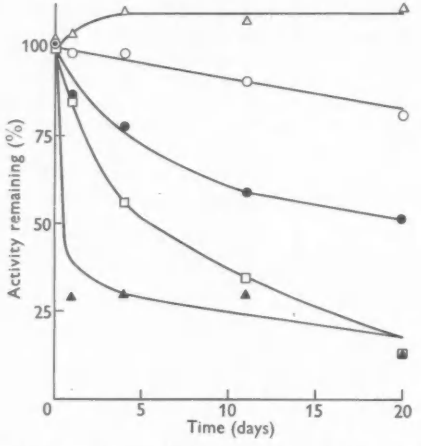

Fig. 3. Protection against copper inactivation at 5°. Xanthine oxidase (0·1%) in 0·04 M-tris–maleate buffer (pH 7·0) was treated with 0·1 mM-$CuSO_4$ (apart from control without copper). The samples were as follows: ○, control without copper or additive; △, 0·01 M-EDTA; ●, 0·01 M-salicylate; □, 0·04 M-benzoate; ▲, no additive.

Table 2. *Effect of salicylate on copper inactivation of xanthine oxidase*

The solutions were incubated for 24 hr. at 37° with 0·1% xanthine oxidase in 0·8 M-phosphate buffer, pH 5·8.

Concn. of $CuSO_4$ (mM)	Concn. of salicylate (mM)	Activity remaining (%)
—	—	53
—	1	76
0·03	—	14
0·03	1	77

Table 3. *Effect of ascorbate on copper inactivation of xanthine oxidase*

The solutions were incubated for 24 hr. at 0° with 0·1% xanthine oxidase in 0·1 M-tris–maleate buffer, pH 7.

Concn. of $CuSO_4$ (mM)	Concn. of ascorbic acid (mM)	Activity remaining (%)
0·1	—	43
0·1	1	4*
—	1	80
0·1	1 (EDTA 10 mM)	97

* Activity (%) before adding the reagents, rather than after, as the activity fell very rapidly on adding Cu^{2+} ions.

Table 4. *Effects of redox buffers on copper inactivation of xanthine oxidase*

The solutions were incubated for 3 hr. at 0° with 0·1% xanthine oxidase in 0·04 M-tris–maleate buffer (pH 7), containing 0·04 mM-$CuSO_4$ and 0·3% of cetyltrimethylammonium bromide (Cetavlon) (to increase the solubility of the redox pairs).

Redox pair (each mM)	E'_0	Activity remaining (%) Without Cu*	With Cu†
Nil	—	105	64
Quinone–quinol	+0·28	112	57
Naphthaquinone–naphthaquinol	+0·04	107	26
Dehydroascorbic–ascorbic acid	+0·06	94	25

* Relative to control without $CuSO_4$ and Cetavlon.
† Remaining activity as percentage of that in previous column.

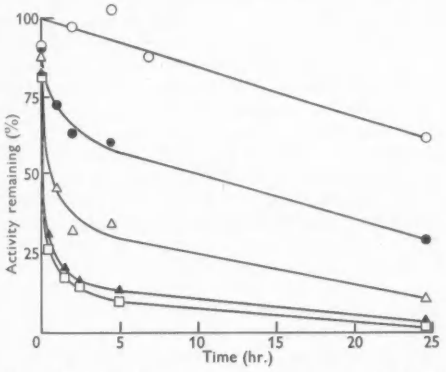

Fig. 4. Inactivation curves with varying concentrations of copper. Decomposition of xanthine oxidase (0·1%) at 37°, in 0·1-M-tris–maleate buffer, pH 6·9. Activities are expressed as a percentage of the extrapolated value at zero time for the control without copper; the first assay was carried out immediately after mixing at 20°. Concentrations of $CuSO_4$ were as follows: ○, nil; ●, 10 μM; △, 25 μM; ▲, 0·1 mM; □, 0·25 mM.

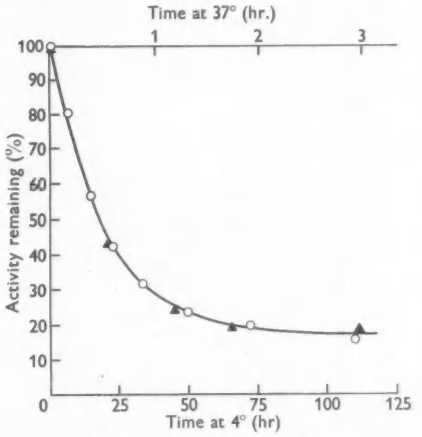

Fig. 5. Copper inactivation at 4° and 37°. Xanthine oxidase (0·1%) was incubated in 0·8 M-phosphate buffer (pH 6), with 0·1 mM-$CuSO_4$. Activities are expressed as a percentage of those before adding copper. ○, Solution at 37°; ▲, solution at 4°. The activities of controls without copper were: 94% after 112 hr. at 4° and 87% after 3 hr. at 37°.

That the effect of ascorbic acid was due to reduction of the metal to the Cu^+ state, which must be therefore a more potent inactivator than the Cu^{2+} ion, was demonstrated in experiments with redox buffers (Table 4). Cu^{2+}/Cu^+ has $E'_0+0·16$ (Anderson & Plaut, 1949). It is thus not surprising that dehydroascorbic–ascorbic acid and 1:4-naphthaquinone–1:4-naphthaquinol, both having lower and very similar redox potentials (cf. Anderson & Plaut), should produce a greater loss of activity, in contrast with benzoquinone–quinol, which would be expected to maintain the metal in its higher-valency state.

The kinetic aspects of the inactivation reaction by copper are illustrated in Figs. 4 and 5. Fig. 4, showing the effects of different concentrations of metal on the course of reaction, discloses the existence of rapid and slow phases. The extent of the former depended on the concentration of copper employed, while the latter was not influenced by the metal. A change of temperature from 37° to 4° reduced the rate of the first phase of reaction 40-fold (Fig. 5) without affecting the activity remaining at the end of this phase.

In order to confirm that inactivation by copper was taking place during the incubation period and not in the assay, the effect of varying the volume of the xanthine oxidase–copper mixture used in the assay, after 24 hr. incubation in 0·8 M-phosphate (pH 6), with 0·1 M-$CuSO_4$, was studied. The activities were found to be the same whether 50-, 100- or 250-fold dilution was employed for the assay.

Treatment of xanthine oxidase with copper, like many other procedures for destroying the

purine oxidase activity of the enzyme (Avis, Bergel & Bray, 1956; Bergel & Bray, 1958), had no effect on the activity towards DPNH as substrate. This is illustrated by the data in Table 5; these were obtained by measuring the diaphorase-like activity with ferricyanide as electron acceptor, a procedure devised by V. Massey (personal communication) which gives considerably higher absolute activities than do other methods (cf. Avis, Bergel & Bray, 1956). In fact the absolute activities in the two assay systems in the experiment reported in Table 5 were approximately equal before the copper was added.

Table 5. *Effect of copper on diaphorase activity of xanthine oxidase*

The solutions were incubated for various times at 0–2°, with 0·1% xanthine oxidase in 0·06M-tris–maleate buffer (pH 7·0), with 0·1 mM-$CuSO_4$.

Time (hr.)	Xanthine–O_2 activity (%)	DPNH–$Fe(CN)_6^{3-}$ activity (%)
0·3	92	98
2·3	77	100
27	55	96
432	39	100

We have studied the effects of sodium *p*-chloromercuribenzoate (*p*CMB) in the absence of substrate, and obtained results which indicated a lower reactivity of crystallized xanthine oxidase towards this reagent than was observed by either Harris & Hellerman (1956) or Fridovich & Handler (1958*a*). No change in the visible colour of xanthine oxidase solutions resulted from our treatment with *p*CMB. The enzyme differed in this respect from succinic dehydrogenase (Massey, 1958). We have not been able to reverse *p*CMB inhibition of xanthine oxidase after prolonged incubation, with either EDTA or glutathione.

Fig. 6 shows that with our highly purified preparation of xanthine oxidase nine equivalents of *p*CMB (expressed as a number of SH groups reacting, calculated from $\Delta\epsilon_{250\ m\mu}$ $7{\cdot}6 \times 10^3$; Boyer, 1954) combined without causing any loss of activity of the enzyme. This contrasts with experiments by Fridovich & Handler (1958*a*) in which eight equivalents, in the absence of the substrate (as in our experiments), produced considerable inactivation.

Our results in Fig. 7 show that with a large amount of the organo-metal derivative ([*p*CMB]/[xanthine oxidase] = 105) substantial inactivation

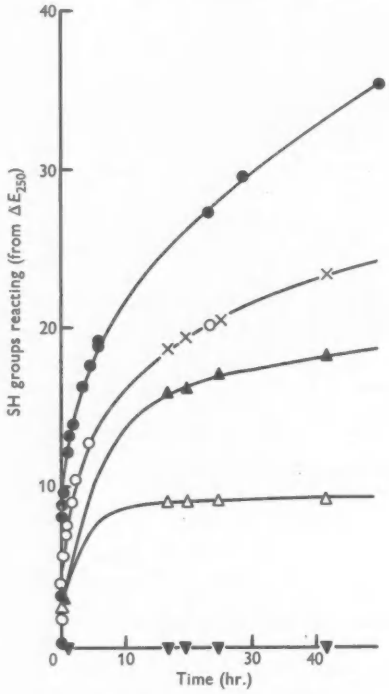

Fig. 6. Reaction of SH groups of crystallized xanthine oxidase with varying molar proportions of *p*CMB. A constant xanthine oxidase concentration of 0·075% (i.e. 2·5 μM) in 0·1M-pyrophosphate buffer (pH 8) was used at 24°. ▼, Xanthine oxidase alone; △, [*p*CMB]/[XO] = 9; ▲, [*p*CMB]/[XO] = 17; ○, ×, [*p*CMB]/[XO] = 26 (two expts.); ●, [*p*CMB]/[XO] = 105. Activity figures (relative to controls containing no *p*CMB, after 20–70 hr.) were respectively 100, 84, 46 and 4% at [*p*CMB]/[XO] ratios of 9, 17, 26 and 105.

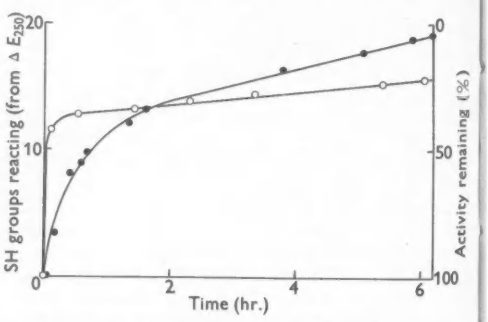

Fig. 7. Number of SH groups reacting and activity, with [*p*CMB]/[XO] = 105. The data on the number of groups reacting is the same as that in Fig. 6. The activity was calculated relative to a control without *p*CMB. The reaction mixture was diluted to a *p*CMB concentration of 10 μM for the activity measurements; the extent of dilution before assay was not critical. ○, Percentage activity (ordinate on right); ●, SH groups reacting.

occurred when the changes in extinction indicated that only a very few SH groups could have reacted.

Inactivation by hydrogen peroxide, peracetic acid and by photo-oxidation at 37°

Hydrogen peroxide and peracetic acid. It has been reported by Siegel & Weintraub (1952) that their relatively crude and dilute samples of xanthine oxidase were substantially inactivated by H_2O_2, even at concentrations of the reagent as low as 0·1 mM. In contrast, we have found that purified xanthine oxidase in relatively concentrated solution at 0–5° was much more resistant to the oxidizing agent. Table 6 shows that even with M-H_2O_2 the inactivation process took several hours, while 0·01 M-H_2O_2 had little effect unless the enzyme was diluted considerably. As the inactivation with M-H_2O_2 did not produce precipitation or change of colour, we have used this procedure to prepare an inactive form of xanthine oxidase (cf. Bergel & Bray, 1958). On the other hand, peracetic acid could not be used for this purpose because, although mM-solutions of the reagent gave rapid inactivation, this was accompanied by loss of colour, indicating a breakdown of the xanthine oxidase molecule.

Photo-oxidation at 37°. Although storage of enzyme solutions for two days at 37°, without access to air or under nitrogen or in complete darkness, caused some loss of their activity, both oxygen and light had a marked accelerating influence on the inactivation process (Table 7). This was not accompanied by any significant precipitation of denatured protein. With larger volume samples the fall in activity slowed down, or even stopped, after some hours unless the solutions were stirred to increase the access of air.

Table 6. *Effect of hydrogen peroxide on xanthine oxidase*

The solutions were incubated for various times at 0–5° with the reagent, in M-phosphate buffer, pH 6.

Concn. of H_2O_2 (M)	Xanthine oxidase (%)	Time (hr.)	Activity remaining (%)
0·01	0·4	53	92
0·01	0·04	53	75
0·01	0·004	53	7
1·0	0·4	—	84
1·0	0·4	1·5	38
1·0	0·4	19	0

Thermal denaturation at 56°

It has been mentioned above that xanthine oxidase solutions could lose their activity without precipitation or other changes in the physicochemical properties of the metalloflavoproteins (cf. Avis, Bergel, Bray, James & Shooter, 1956) but with a decrease in activity/E_{450} ratio. However, when inactivation was carried out at 56°, an arbitrarily chosen temperature high enough to achieve a speedy loss of activity, this process was largely one of denaturation. Thus when a sample of xanthine oxidase at a concentration of 0·9 % was kept at this temperature in 0·1 M-phosphate buffer (pH 6) for 4·5 hr., considerable precipitation occurred and the suspension retained 57 % of the original activity. The mixture was cooled and, in order to remove any free flavin derivatives, was dialysed at 5°, side by side with, as a control, a sample of the starting solution which had been diluted with buffer to give the same activity as the heated sample. After centrifuging, the supernatant from the heated and unheated samples had activity/E_{450} of 52 and 55 respectively. This meant that, while the overall activity of the heated sample had fallen by 43 %, its activity/E_{450} ratio had decreased by only 6 %. Thus almost all of the activity loss was attributable to gross denaturation, which was accompanied by removal of material absorbing at 450 mμ, either by centrifuging or by the dialysis.

The course of the inactivation reaction at 56° is shown in Figs. 8*a* and *b*. Under a variety of conditions the activity was found to fall rather rapidly by 20–35 % on placing the solutions in the thermostat; after this the rate of loss of activity decreased, and subsequently the inactivation process followed unimolecular kinetics fairly closely. In

Table 7. *Loss of xanthine oxidase activity at 37°*

The solutions were incubated for 48 hr. under ordinary laboratory lighting conditions (except for the second experiment), with 0·6 % xanthine oxidase, in M-phosphate buffer, pH 6.

Conditions		Vol. of xanthine oxidase (ml.)	Activity remaining (%)
Standard	With air above solutions	0·2	10
Wrapped in aluminium foil	With air above solutions	0·2	39
Standard	With air above solutions	1·0	29
No air space	With air above solutions	1·0	74
Oxygen	First bubbled through buffer, then passed over samples for rest of time	0·5	32
Nitrogen	First bubbled through buffer, then passed over samples for rest of time	0·5	85

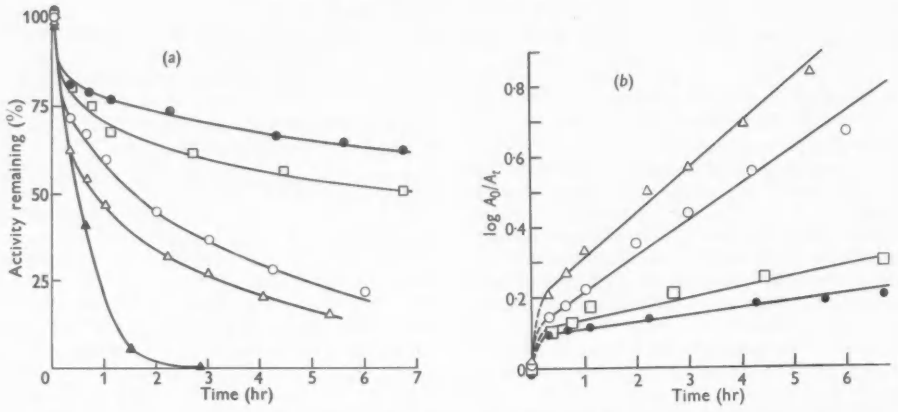

Fig. 8. Decomposition of xanthine oxidase at 56°. The concentration of xanthine oxidase was 0·4% in 0·1 M-phosphate buffer (pH 6·0) containing 0·1 M-NaCl. A_0/A_t = Activity at start/activity at time t. ○, In darkness, under N_2, with 0·01 M-EDTA; all other samples were without exclusion of air and under ordinary laboratory lighting conditions; ▲, no additions; △, 0·01 M-EDTA; □, 0·01 M-EDTA and 0·05 M-sodium benzoate; ●, 0·01 M-EDTA and 0·01 M-sodium salicylate.

Table 8. *Loss of xanthine oxidase activity at* 56°

The concn. of xanthine oxidase was 0·1% for the four experiments, which were carried out at different times on the same enzyme preparation.

Buffer	Additives	Activity remaining (%)
Incubated for 15 hr., at pH 7, with 0·03 M-salicylate		
0·1 M Phosphate	—	37
0·5 M Phosphate	—	43
0·9 M Phosphate	—	50
0·1 M Phosphate	1·1 M-$(NH_4)_2SO_4$	47
0·1 M Phosphate	1·1 M-NaCl	33
0·1 M Phosphate	3·2 M-NaCl	21
Incubated for 1 hr., at pH 6		
0·1 M-Tris–maleate	—	74
0·1 M-Acetate	—	67
0·1 M-Phosphate	—	58
0·1 M-Pyrophosphate	—	55
Incubated for 3 hr., at pH 7		
0·9 M-Phosphate	—	32
0·9 M-Phosphate	0·01 M-Sulphosalicylate	29
0·9 M-Phosphate	0·01 M-Gentisate	32
0·9 M-Phosphate	0·01 M-*p*-Aminosalicylate	46
0·9 M-Phosphate	0·01 M-Benzoate	51
0·9 M-Phosphate	0·01 M-Salicylate	83
Incubated for 3 hr., at pH 7		
0·9 M-Phosphate	—	24
0·9 M-Phosphate	0·01 M-EDTA	40
0·9 M-Phosphate	0·01 M-Salicylate	63
0·9 M-Phosphate	0·01 M-Salicylate; 0·01 M-EDTA	77
0·9 M-Phosphate	0·04 M-Benzoate	51
0·9 M-Phosphate	0·04 M-Benzoate; 0·01 M-EDTA	75

other experiments, which are not reported in detail, decomposition curves of the same general shape as those in Figs. 8*a* and *b* were obtained with a number of different samples of xanthine oxidase. The form of these inactivation curves remained unchanged at various concentrations of xanthine oxidase, although diluting the enzyme resulted in an increased rate of decomposition (cf. Fig. 11). Also partial inactivation at 56° of the sample, discussed at the beginning of this section, with subsequent dialysis and centrifuging, did not produce a disappearance of the characteristic initial rapid drop in the activity of the solution (cf. Figs. 8*a* and *b*).

Table 8 shows the effect of inorganic salts and of various other compounds on the stability at 56°, at pH 6 or 7. The effects of salts were relatively small, though in the presence of sodium salicylate high concentrations of phosphate had a slight stabilizing effect, while sodium chloride seemed to decrease stability somewhat. Salicylate increased the stability at 56° substantially; sodium benzoate and *p*-aminosalicylate also had some effect, but sulphosalicylate and gentisate were without effect. EDTA had a slight protecting action and this was to some extent additive to that given by salicylate or benzoate.

The effect of varying the concentration of salicylate on the stability of xanthine oxidase is illustrated in Fig. 9, and that of pH in Fig. 10. The region of optimum stability was pH 6–8 in pyrophosphate buffer or in tris–maleate buffer, with or without salicylate. The latter had little stabilizing action in tris–maleate, though it was effective in pyrophosphate.

As expected, decomposition was more rapid in dilute solutions than in concentrated solutions of the enzyme (Fig. 11). Maintaining a constant protein concentration by adding albumin, or introducing a non-ionic detergent (Tween 80), did not affect the results of such experiments.

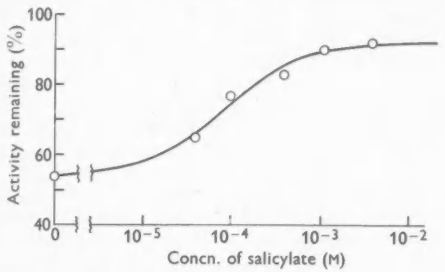

Fig. 9. Decomposition of xanthine oxidase with varying concentrations of sodium salicylate, during 1 hr. at 56°, in 0·9 M-phosphate buffer, pH 6. The theoretical curve through the experimental points was calculated assuming a dissociation constant for the xanthine oxidase–salicylate complex of 80 μM (see Experimental).

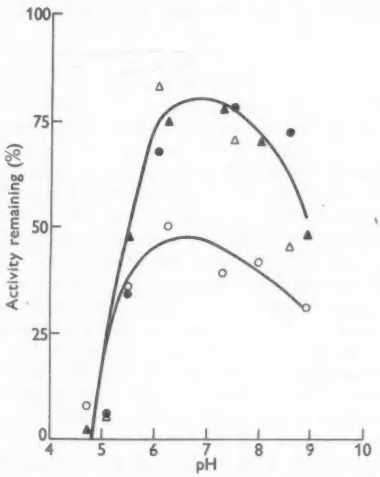

Fig. 10. Effect of pH on decomposition of xanthine oxidase during 1 hr. at 56°. ○, 0·1 M-Pyrophosphate buffer; ▲, 0·1 M-pyrophosphate with 0·03 M-salicylate; △, 0·1 M-tris–maleate buffer; ●, 0·1 M-tris–maleate buffer with 0·03 M-salicylate.

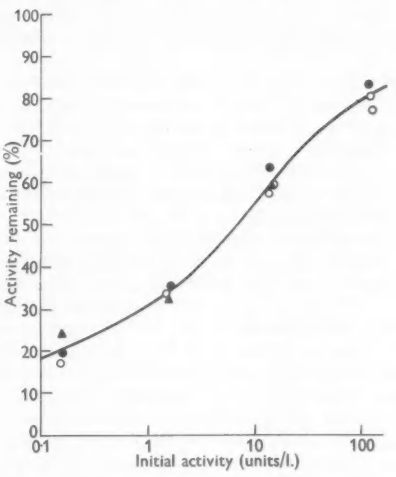

Fig. 11. Effect of enzyme concentration on decomposition during 1 hr. at 56° in 0·1 M-pyrophosphate buffer, pH 8. ○, No additions; ▲, solutions containing 1% of albumin; ●, solutions containing 0·05% of Tween 80. (For definition of activity units, see Avis *et al.* 1955.)

DISCUSSION

Metal inactivation

The experiments carried out with copper give no definite information on the groups of the xanthine oxidase molecule which interact with the metal. However, the facts that the inactivation is reversible under some conditions, at least, by EDTA, and that the Cu^{+} ion is a more potent inactivator than the Cu^{2+} ion, suggest a reaction with a sulphur-containing group of the enzyme. Harris & Hellerman (1956) have proposed, after experiments with *p*CMB and other reagents, that xanthine oxidase has SH groups essential for its activity. Fridovich & Handler (1958*a*, *b*) found that *p*CMB inactivated the enzyme much more rapidly in the presence than in the absence of hypoxanthine, and from this and other results concluded that the active site of xanthine oxidase may contain a metal (Fe or Mo) mercaptide group which was converted by the substrate into an SH group and the reduced form of the metal (cf. Bray, Malmström & Vänngård, 1959). In our experiments with crystallized xanthine oxidase and *p*CMB there was very little correlation between inhibition and the apparent number of SH groups blocked. It seems that either the reagent was inactivating by reaction with a non-sulphur-containing group (cf. Rabinovitch & Barron, 1955) or, if one accepts the presence of a metal mercaptide, that high concentrations at least of *p*CMB could compete with the metal of the enzyme for the sulphur in the absence of the substrate. In the latter case, the mechanism of copper reaction with xanthine oxidase was possibly analogous to that with *p*CMB. Consequently the failure to attain complete interaction with copper under some of our conditions (Figs. 4, 5) might have been due to an equilibrium state between active enzyme with iron or molybdenum mercaptide groups and inactivated enzyme containing copper mercaptides.

The effects of the inorganic salts and other reagents on copper inactivation probably reflected changes in the availability of the inactivating metal ion which was partially removed by 'complexing' or chelation.

Whereas in our experiments ascorbic acid alone caused only slight inactivation (Table 3), Feigelson (1952) reported on the inhibitory effect of this vitamin on his xanthine oxidase concentrate. In view of our own observations and those by Mapson (1946) on urease and Ito & Abe (1953) on β-amylase, it seems logical to conclude that the phenomenon reported by Feigelson was due to reduction of traces of copper present in his sample to Cu^{+} ions, which then inactivated his xanthine oxidase preparation.

Oxidative inactivation

Inactivation by hydrogen peroxide, formed during the enzymic reaction, has been commented on previously (e.g. Dixon, 1925).

The mechanism of inactivation by added hydrogen peroxide in our experiments may have been analogous to that occurring at 37°, which was essentially a photo-oxidation. In both these reactions the enzyme lost its activity, but remained almost entirely in solution without any substantial decrease in E_{450}. Other workers have reported on the inactivation of xanthine oxidase in homogenates or crude preparations by oxygen (Stadie & Haugaard, 1945; Mann & Quastel, 1946) and by light (Siegel & Weintraub, 1952). The photo-oxidation of a number of other enzymes has been studied in some detail by Polis & Shmukler (1953), Weil, James & Buchert (1953) and Weil & Seibler (1955). We found no evidence suggesting that the photo-oxidation process could be reversed *in vitro*, though reversal *in vivo* may be possible (cf. Haddow *et al.* 1958). Whether photo-oxidation liberates any of the co-factors from xanthine oxidase or reduces the firmness of their binding to the protein is a question which has not yet been investigated.

The temperature coefficient of a photo-oxidation reaction would be expected to be lower than that of a thermal denaturation; hence it was not surprising that at 56° the latter reaction, which was accompanied by precipitation of the enzyme protein and by liberation of the flavin, predominated.

Stabilization by competitive inhibitors and metal-binding agents

The phenomenon of protection of enzymes by competitive inhibitors may be general and has been studied in detail with D-amino acid oxidase by Burton (1951), Vietch & McComb (1956) and Klein (1957). Sodium salicylate was a competitive inhibitor of xanthine oxidase with K_i 0·135 mM (Bergel & Bray, 1956). The similarity between this value and the dissociation constant for the postulated xanthine oxidase–salicylate complex, as calculated from the stability experiment at 56° reported in Fig. 9, namely 0·08 mM, might be taken as evidence that the salicylate did in fact stabilize by combining with the enzyme. However, the temperature, pH and salt concentration at which the two sets of data were obtained were substantially different; also there is some uncertainty as to the exact Michaelis constant of xanthine oxidase. An alternative possibility is that salicylate might stabilize, particularly in the experiments at 56°, by chelating trace-metal impurities; but the observation that this compound still had a beneficial effect even in the

presence of the much more powerful chelator, EDTA (Figs, 8*a*, *b*; Table 8), provided additional evidence that some of the effects of salicylate were due to enzyme–stabilizer interaction. On the other hand, in the experiments on copper inactivation, it is probable that salicylate was acting predominantly by chelation (i.e. inactivator–stabilizer interaction), as it could with advantage be replaced by EDTA under these conditions (Fig. 3). In the studies reported by Bergel & Bray (1956), salicylate stabilized the enzyme at 37°, but EDTA was without effect. The latter result indicates that trace-metal inactivators were absent in those early experiments; it thus seems that, as in thermal denaturation at 56°, salicylate stabilized the enzyme against photo-oxidation at 37° by combination with it.

The other two substances found to stabilize the enzyme in the present experiments were sodium benzoate and *p*-aminosalicylate. The latter has been reported by Di Fonzo (1952), to be an inhibitor of xanthine oxidase, and we have established the inhibitory action of benzoate, with K_i 1·5 mM measured under the conditions used previously and assuming the same K_s (Bergel & Bray, 1956). Thus the action of these two substances was probably entirely analogous to that of sodium salicylate, though the higher dissociation constant of the benzoate meant that it had to be employed in higher concentrations than salicylate to obtain comparable stabilization (Table 8). The anti-copper action of benzoate of course depended, not on chelation, but possibly on a low dissociation of the copper benzoate [cf. silver benzoate (Bjerrum, Schwarzenbach & Sillén, 1957)]. Sulphosalicylate and gentisate had no effects in these experiments, although we reported previously (Bergel & Bray, 1956) on their low stabilizing action at 37°.

Fried (1958) has reported that inorganic phosphate could exert both protecting and restoring actions on xanthine oxidase activity. Our experiments at 56° (Table 8) confirmed that phosphate exerted a slight protecting action, and preliminary experiments suggested that it might have a greater effect on the reaction at 37°, though this finding awaits confirmation.

Kinetics of thermal inactivation

The reason why the inactivation reaction at 56° did not follow strictly unimolecular kinetics is not clear. Prostatic acid phosphatase at 50–56° (London, Wigler & Hudson, 1954) and carboxypeptidase at 45–55° (Labouesse, 1957) have both been reported to decompose according to unimolecular kinetics. The data in Figs. 8*a* and *b*, however, suggest that the characteristic break in the curves was not due to a superimposition of photo-oxidation or metal inactivation on the thermal-denaturation reaction. A possible explanation of the phenomenon relates to the formation of an unknown inhibitor–stabilizer during denaturation of xanthine oxidase (R. C. Bray, unpublished work).

Surface denaturation seemed unimportant in the decomposition of xanthine oxidase at 56°, as albumin and a non-ionic detergent, which have been found to protect enzymes sensitive to surface denaturation at low concentrations, afforded no protection (Fig. 11) (cf. Labouesse, 1957; Tsuboi & Hudson, 1955).

Conditions for purification, storage and utilization

In the handling and storage of solutions of xanthine oxidase, one has to guard against all three inactivation mechanisms, namely inactivation by trace-metal contaminants, photo-oxidation and gross denaturation. In addition to these reactions, it is also apparently possible for the flavin to be split off without precipitation of the apo-enzyme under certain conditions (cf. Morell, 1952), though we have not studied this reaction. The evidence presented above shows that it should be possible to obtain some control over the three reactions by suitable additions of chelating agents and competitive inhibitors, and by protecting solutions of xanthine oxidase from oxygen and light.

Sodium salicylate is a particularly useful stabilizer, as it combines chelating and inhibitory properties. The value of its K_i is such that above concentrations of about mM it gave maximum stabilization (Fig. 9), while below concentrations of about 0·1 mM it did not cause significant inhibition in the ordinary activity assays. Enzyme solutions, stabilized by salicylate, could thus be assayed without prior dialysis, dilution alone being adequate to reverse inhibition.

As mentioned by Bergel & Bray (1956) it is convenient to add salicylate to xanthine oxidase samples during purification and storage. Batches of xanthine oxidase have been prepared and crystallized by the original method (Avis *et al.* 1955) with mM-stabilizer present at all stages, with good results. The conditions generally chosen for storing the enzyme have been as an approx. 1% solution just above freezing-point in M-sodium and potassium phosphate buffer (pH 6), containing 0·03M-sodium salicylate. Although these conditions might perhaps be improved on, according to the results presented above, they have generally given xanthine oxidase solutions which have lost activity at a rate of less than 5% per month, while in some cases activity has remained unchanged for more than two years.

SUMMARY

1. The loss of activity of solutions of xanthine oxidase has been studied under a variety of conditions in the presence of copper salts, *p*-chloromercuribenzoate, hydrogen peroxide and at 37° and 56°.

2. At least three mechanisms by which activity is likely to be lost have been distinguished. These mechanisms are heavy-metal inactivation, photo-oxidation and gross denaturation.

3. The effect of reducing agents (especially ascorbic acid) on inactivation by copper has been studied and is discussed in relation to the reported inhibitory action of ascorbic acid on xanthine oxidase.

4. Chelating and 'complexing' agents protected against metal inactivation.

5. Competitive inhibitors protected against photo-oxidation and gross denaturation.

6. Sodium salicylate (which may function both as an inhibitor and a chelating agent) has been found to be a particularly useful protecting agent during manipulation and storage of xanthine oxidase.

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REFERENCES

Anderson, L. & Plaut, G. W. E. (1949). In *Respiratory Enzymes*, p. 71. Ed. by Lardy, H. A. Minneapolis, U.S.A.: Burgess Publishing Co.

Avis, P. G., Bergel, F. & Bray, R. C. (1955). *J. chem. Soc.* p. 1100.

Avis, P. G., Bergel, F. & Bray, R. C. (1956). *J. chem. Soc.* p. 1219.

Avis, P. G., Bergel, F., Bray, R. C., James, D. W. F. & Shooter, K. V. (1956). *J. chem. Soc.* p. 1212.

Bergel, F. (1959). *Acta Un. int. Cancr*, (in the Press).

Bergel, F. & Bray, R. C. (1956). *Nature, Lond.*, **178**, 88.

Bergel, F. & Bray, R. C. (1958). *Symp. biochem. Soc.* **15**, 64.

Bergel, F., Bray, R. C., Haddow, A. & Lewin, I. (1957). *Ciba Foundation Symp., The Chemistry and Biology of Purines*, p. 256. London: J. and A. Churchill Ltd.

Bjerrum, J., Schwarzenbach, G. K. & Sillén, L. G. (1957). *Stability Constants*, p. 52, part I. London: The Chemical Society.

Boyer, P. D. (1954). *J. Amer. chem. Soc.* **76**, 4331.

Bray, R. C., Malmström, B. G. & Vänngård, T. (1959). *Biochem. J.* **73**, 193.

Burton, K. (1951). *Biochem. J.* **48**, 458.

Di Fonzo, M. (1952). *Boll. Soc. ital. Biol. sper.* **32**, 955. Quoted in *Chem. Abstr.* **48**, 754.

Dixon, M. (1925). *Biochem. J.* **19**, 507.

Feigelson, P. (1952). *J. biol. Chem.* **197**, 843.

Fridovich, I. & Handler, P. (1958*a*). *J. biol. Chem.* **231**, 899.

Fridovich, I. & Handler, P. (1958*b*). *J. biol. Chem.* **233**, 1581.

Fried, R. (1958). *Experientia*, **14**, 173.

Gomori, G. (1955). In *Methods in Enzymology*, vol. 1, p. 138. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Green, A. A. (1933). *J. Amer. chem. Soc.* **55**, 2331.

Greenspan, F. P. (1946). *J. Amer. chem. Soc.* **68**, 907.

Haddow, A., de Lamirande, G., Bergel, F., Bray, R. C. & Gilbert, D. A. (1958). *Nature, Lond.*, **182**, 1144.

Harris, J. & Hellerman, L. (1956). In *Inorganic Nitrogen Metabolism*, p. 565. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.

Ito, M. & Abe, M. (1953). *J. agric. chem. Soc. Japan*, **27**, 486. Quoted in *Chem. Abstr.* **48**, 5897.

Klein, J. R. (1957). *Arch. Biochem. Biophys.* **67**, 423.

Labouesse, J. (1957). *Biochim. biophys. Acta*, **26**, 52.

London, M., Wigler, P. & Hudson, P. B. (1954). *Arch. Biochem. Biophys.* **52**, 236.

Mann, P. J. G. & Quastel, J. H. (1946). *Biochem. J.* **40**, 139.

Mapson, L. W. (1946). *Biochem. J.* **40**, 240.

Massey, V. (1958). *Biochim. biophys. Acta*, **30**, 500.

Morell, D. B. (1952). *Biochem. J.* **51**, 657.

Philpot, F. J. (1938). *Biochem. J.* **32**, 2013.

Polis, B. D. & Shmukler, H. W. (1953). *J. biol. Chem.* **201**, 475.

Rabinovitch, M. & Barron, E. S. G. (1955). *Biochim. biophys. Acta*, **18**, 316.

Siegel, S. M. & Weintraub, R. L. (1952). *Biochem. J.* **52**, 324.

Stadie, W. C. & Haugaard, N. (1945). *J. biol. Chem.* **161**, 181.

Tsuboi, K. K. & Hudson, P. R. (1955). *Arch. Biochem. Biophys.* **55**, 191, 206.

Vietch, F. P. & McComb, R. (1956). *J. Amer. chem. Soc.* **78**, 1363.

Weil, L., James, S. & Buchert, A. R. (1953). *Arch. Biochem. Biophys.* **46**, 266.

Weil, L. & Seibler, T. S. (1955). *Arch. Biochem. Biophys.* **54**, 368.

The Chemistry of Xanthine Oxidase

5. ELECTRON-SPIN RESONANCE OF XANTHINE OXIDASE SOLUTIONS*

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The participation of metals in the action of metalloflavoproteins is the subject of much controversy, and it has recently been concluded by Singer & Massey (1957) that only for nitrate reductase has strong evidence been presented for valency changes in the metal in the course of the enzymic reactions.

Studies on the function of the molybdenum, iron and flavinadenine dinucleotide of xanthine oxidase are handicapped by the firmness of their binding to the apoenzyme. There is some suggestion of correlation between the molybdenum content of the purified enzyme and its purine/oxygen activity (Avis, Bergel & Bray, 1956; Avis, Bergel, Bray & Shooter, 1956; Bergel & Bray, 1958; Richert & Westerfeld, 1954; Westerfeld, Richert & Higgins, 1956). Reports that molybdenum can be removed reversibly from the enzyme (De Renzo, Heytler & Kaleita, 1954; Mackler, Mahler & Green, 1954) appear to rest on somewhat inadequate evidence (Avis, Bergel, Bray & Shooter, 1956; Westerfeld *et al.* 1956), and do not agree as to which enzyme-catalysed reactions are affected by loss of the metal. According to Totter & Comar (1956), iron is in the ferrous state whether the substrate is present or absent, but the colorimetric method used cannot provide conclusive evidence of this (cf. Singer & Massey, 1957). On the other hand, Fridovich & Handler (1958*a*, *b*) give indirect evidence for the presence of a metal–sulphur bond in the enzyme which is supposed to be split by the substrate to the reduced metal and a thiol group; they presume that the metal involved is iron, but most of their data could be explained equally well on the basis of a molybdenum–sulphur bond (P. Handler, personal communication, 1958). It appears that the flavinadenine dinucleotide analysis, on which much of their argument for implicating iron rather than molybdenum in this scheme depends, may be high by a factor of 1·71 (Morell, 1952; P. Handler, personal communication, 1958); if an appropriate correction is applied, it seems to the present authors that molybdenum should replace iron in this formulation before it could be accepted.

It is generally agreed that the flavinadenine dinucleotide of xanthine oxidase is alternately reduced by the substrate and oxidized by the electron acceptor (cf. Gutfreund & Sturtevant, 1959), though the spectral changes accompanying this reaction are complicated (Morell, 1952). Considerable evidence from electron-spin resonance studies (Ehrenberg & Ludwig, 1958; Commoner, Lippincott & Passonneau, 1958) and other experiments (Beinert, 1956, 1957) has recently suggested that flavin free radicals may be intermediates in catalysis by a number of flavoprotein enzymes. However, xanthine oxidase has not been included in any of these studies.

The technique of electron-spin resonance (Wertz, 1955; Ingram, 1958) allows the study of ions or molecules containing unpaired electrons, e.g. free radicals and ions of transition metals. The sample to be examined is placed in a static magnetic field which is varied continuously, while at right-angles to this field electromagnetic radiation of a fixed radio-frequency is applied; absorption of energy from the radio-frequency oscillation by the sample is measured while the magnetic field strength is varied. A simple explanation of the resonance phenomenon may be given by likening each unpaired electron in the sample to a spinning top. The rotating electron acts as a magnetic dipole and the effect of the static field is to cause precession of the 'gyroscope'. The radio-frequency oscillation may be considered as equivalent to a rotating magnetic field; when the frequency of the rotation coincides with that of the precession, the motion of the electron is affected and energy is absorbed. The condition for this 'resonance absorption' is given by $h\nu = g\beta B$, where h is Planck's constant, ν the frequency of the radio-frequency electromagnetic field (and of the associated rotating magnetic field), β is the Bohr magnetron, B is the strength of the static magnetic field and g is a dimensionless quantity called the 'spectroscopic splitting factor'. For a completely free electron g has the value 2·0023, but deviations from this value, due to binding of the electron, are often observed and the g-value is thus an important factor in interpreting resonance absorption. Other characteristic features are line-width and the so-called 'hyperfine

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structure' which results from a splitting of energy levels, owing to interaction with nuclear spin. The intensity of an electron-spin-resonance absorption signal is related to the number of unpaired electrons in the sample.

Electron-spin resonance thus detects free radicals and transition metals at certain valency levels; under favourable conditions it is capable of distinguishing between these and of estimating their concentrations. Biochemical applications of electron-spin resonance are relatively new, but it has recently been used successfully in a study of the state of copper in laccase by Malmström, Mosbach & Vänngård (1959). In the present work xanthine oxidase solutions treated in various ways have been examined by the method in the hope that it might give reliable evidence on the valency of the metals and on the possible presence of free radicals.

MATERIALS AND METHODS

The xanthine oxidase used for most of the experiments was a sample (stage S4) from the procedure of Avis, Bergel & Bray (1955), further purified by zone electrophoresis on a cellulose column according to the technique of Porath (1954) and Flodin & Kupke (1956). The cellulose was obtained from Grycksbo Pappersbruk, Grycksbo, Sweden, and the buffer (total ionic strength approx. 0·05) for the electrophoresis was tris (2-amino-2-hydroxymethylpropane-1:3-diol)–maleic acid containing ethylenediaminetetra-acetic acid and salicylic acid (Bergel & Bray, 1959). The xanthine oxidase obtained had a ratio of extinctions at 280 and 450 mμ (E_{280}/E_{450}) of 5·5 and appeared to be at least 90% pure in the ultracentrifuge; the ratio activity/E_{450} was about 60. [The ratios E_{280}/E_{450} and activity/E_{450} are referred to as 'PFR' and 'AFR' in previous publications (Avis *et al.* 1955).] The concentration of xanthine oxidase in the solution (0·2 mM) was calculated on the basis of a molar extinction coefficient of 70 000 at 450 mμ (Avis, Bergel, Bray, James & Shooter, 1956; Avis, Bergel & Bray, 1956). The contents of iron, molybdenum and flavinadenine dinucleotide were presumably similar to those reported previously (Avis, Bergel & Bray, 1956). Several of the experiments described below were also repeated with a crystallized sample (Avis *et al.* 1955) of the enzyme.

A sample obtained from a crystallized preparation which had lost almost all of its activity on storage was also used for certain experiments; this had activity/E_{450} = 1 and its concentration was 0·1 mM. It was analysed (after dialysis) for molybdenum by a modification of the procedure of Clark & Axley (1955) by digesting with sulphuric acid and hydrogen peroxide, treating with potassium iodide in hydrochloric acid and removing liberated iodine with an excess of ascorbic acid, then forming the green complex with toluene-3:4-dithiol in the presence of tartaric acid, extracting it into amyl acetate, and measuring at 680 mμ. Despite its low activity, the enzyme sample contained about one atom of molybdenum/mole of xanthine oxidase (cf. Bergel & Bray, 1958).

Solutions were dialysed against phosphate buffer, pH 6 (0·08 M-KH_2PO_4 and 0·02 M-Na_2HPO_4) containing mM-salicylate and mM-ethylenediaminetetra-acetic acid. Samples (approximately 0·15 ml.) were placed in quartz tubes (2·5 mm. internal diameter; 5 mm. outside diameter). Additions of xanthine or dithionite were made if required and the solutions were frozen in liquid air before measurement in a Varian V 4500 3 cm. spectrometer. For anaerobic experiments, the tubes were filled and the contents frozen inside a glove cabinet containing nitrogen (cf. Bergel & Bray, 1959).

RESULTS

Electron-spin resonance results are generally plotted as a curve of the first derivative of the absorption against magnetic-field strength; a single absorption band thus gives rise to a peak above, followed by a peak below, the base line.

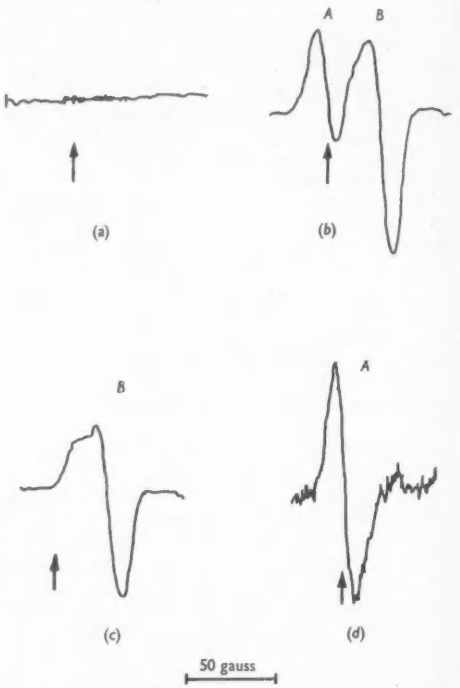

Fig. 1. First derivative of electron-spin-resonance absorption curves of solutions of xanthine oxidase under various conditions. Temperature about 100° K; microwave frequency approx. 9500 Mcyc./sec.; modulation sweep approx. 15 gauss peak-to-peak; magnetic field increasing from left to right. The arrows indicate the resonance field for free electrons. (*a*) Untreated 'active' xanthine oxidase; (*b*) xanthine oxidase shortly after adding xanthine aerobically; (*c*) xanthine oxidase in presence of excess of sodium dithionite; (*d*) 'inactive' xanthine oxidase with a limited amount of sodium dithionite; sensitivity in (*d*) four times that in (*a*)–(*c*).

Untreated xanthine oxidase gave no signal (Fig. 1*a*). When xanthine (approximately 10 moles/mole of enzyme) was added aerobically a signal consisting of two peaks (marked *A* and *B* respectively; Fig. 1*b*) appeared at once and remained unchanged after thawing, shaking to admit more oxygen, and re-freezing. This procedure could be repeated until, at a point presumably corresponding to exhaustion of the substrate, the signal disappeared completely. A similar double signal, though with a slightly different relative intensity of peaks *A* and *B*, appeared on adding xanthine to the enzyme under anaerobic conditions.

In another experiment a solution of the enzyme was titrated anaerobically with sodium dithionite, the solution being frozen for measurement of electron-spin resonance after each addition, then thawed out before the next portion was added. Peaks *A* and *B* appeared simultaneously and increased side by side with small additions of the reducing agent. When an excess of dithionite was present, peak *A* disappeared, leaving only *B* (Fig. 1*c*). If a smaller modulation field was used, peak *B* could be resolved into several components (Fig. 2).

When an 'inactive' sample of xanthine oxidase was treated with substrate, no signals appeared; however, on titration with sodium dithionite a single peak corresponding to *A* was obtained on partial reduction (Fig. 1*d*) and this disappeared, leaving no signal at all, on complete reduction.

A detailed examination of all the electron-spin resonance curves which have been obtained during the course of the work indicated that only two basic types of signal were observed, corresponding to the peaks marked *A* and *B* respectively in Fig. 1. The *g*-values and other features of these signals were accurately reproduced from one experiment to another and from one enzyme sample to another.

DISCUSSION

The most plausible explanation of the results seems to be that peak *A* is due to a flavinadenine dinucleotide semiquinone free radical and peak *B* to a reduced form of molybdenum. The absence of a signal from the resting enzyme indicates that this does not contain significant concentrations of free radicals, or of Fe^{III}, Mo^{V} or Mo^{III}. This conclusion is supported by a preliminary magnetic-susceptibility measurement (Theorell & Ehrenberg, 1951), carried out by Dr A. Ehrenberg, which indicated that the untreated enzyme was diamagnetic to about the extent expected on the basis of the protein content of the solution. These results are thus in agreement with those of Totter & Comar (1956) for the valency of the iron.

Peak *B* must be due to a paramagnetic form of molybdenum or Fe^{II}, since it appears on partial reduction of the enzyme and is still present at full reduction, and hence cannot be due to a free radical or to Fe^{III}. Its *g*-value of $1{\cdot}971 \pm 0{\cdot}002$ at maximum absorption is in the range reported for 3- and 5-valent molybdenum compounds (Griffiths, Owen & Ward, 1953; Weissman & Cohn, 1957), while paramagnetic Fe^{II} compounds would give very broad and weak resonances. It is unfortunately not possible to decide from the available data whether peak *B* is caused by Mo^{III} or by Mo^{V}. However, by analogy with nitrate reductase (Nicholas & Stevens, 1955), Mo^{V} is perhaps more probable. If this is so, it is necessary, in order to explain the four components or more in peak *B* (Fig. 2) (which cannot be due to hyperfine structure), to assume the presence of chemically non-equivalent forms of molybdenum in the enzyme samples. The reason for the absence of peak *B* from the signal of the 'inactive' preparation reduced by sodium dithionite is not certain, as the sample contained molybdenum. Possibly inactivation of the enzyme results in steric changes or a change in the binding of the protein-bound metal, so that the molybdenum ceases to be affected, or is affected in a different manner, by the reducing agent.

Peak *A*, which appears on partial reduction and disappears on complete reduction of xanthine oxidase, has a *g*-value of $2{\cdot}004 \pm 0{\cdot}001$, and a linewidth of 18 gauss. These figures are identical with the values in the literature for free radicals from riboflavin compounds (Ehrenberg & Ludwig, 1958;

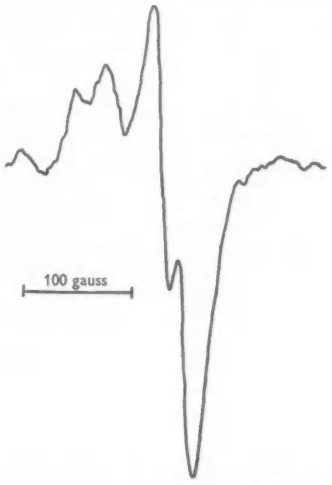

Fig. 2. Electron-spin resonance signal of the sample used for Fig. 1 (*c*), recorded with a smaller modulation field (approx. 5 gauss peak-to-peak).

cf. Commoner & Lippincott, 1958); similar signals were also obtained here from riboflavin reduced by means of zinc and hydrochloric acid. There has been one report of a Mo^{V} compound giving a g-value of 2·005 (Griffiths *et al.* 1953), and this might suggest that the data could be interpreted by assigning peak *A* to a reduced form of molybdenum. However, the appearance of this peak on partial reduction and its disappearance on complete reduction, particularly with the 'inactive' sample where there is no complication from the presence of peak *B*, makes it highly probable that peak *A* is in fact due to a free radical. Since the signal corresponding to peak *A* is the same whether reduction is carried out with xanthine or with sodium dithionite, the free radical seems to be associated with the enzyme rather than with the reducing agent, and it is most likely that it is due to a semiquinone flavinadenine dinucleotide free radical. All semiquinones do, in fact, have almost identical g-values (Adams, Blois & Sands, 1958).

Comparison of the intensity of the peak *A* signal with that from a solution of diphenylpicrylhydrazyl in benzene suggested that, in the presence of the substrate and of oxygen, about 15% of the total flavin was present as the free radical. However, as the enzyme sample contained a proportion of 'inactive' molecules (Avis, Bergel & Bray, 1956), the percentage of active enzyme flavinadenine dinucleotide in the free-radical form would be higher than this.

It has recently been claimed (Isenberg & Szent-Györgyi, 1958), that a riboflavin–tryptophan free-radical complex is produced on mixing the amino acid with riboflavin derivatives, and that this observation may make it necessary to reinterpret some past work on flavin-free radicals. Nevertheless, in agreement with A. Ehrenberg (1958, personal communication), we found no signal and hence no significant amounts of free radicals in the orange–red riboflavin–tryptophan complex in the absence of a reducing agent. Similarly, no signals were obtained on adding tryptophan to xanthine oxidase. Commoner & Lippincott (1958) obtained free radicals on irradiating a flavoprotein enzyme. Although we have not studied the effect of light on electron-spin resonance signals from xanthine oxidase, the early experiments of Bernheim & Dixon (1928), quoted by the above workers, in which it was claimed that xanthine oxidase was activated by light, could not be repeated by Siegel & Weintraub (1952); the latter found, as confirmed in Part 4 (Bergel & Bray, 1959), that light had an inactivating effect on the enzyme.

The experiments reported herein thus provide good evidence that iron is unaffected, molybdenum changes valency and free radicals are formed when the substrate is added to the enzyme. While it has not actually been shown that these changes are an essential part of the enzymic reactions, they seem to provide the strongest presumptive evidence which is yet available that molybdenum and also flavinadenine dinucleotide free radicals participate in the oxidation–reduction sequences of xanthine oxidase action.

Since the work reported in this paper was completed, E. Ackerman & A. Brill (personal communication; paper presented at Pittsburgh, Pa., U.S.A., on 25 February, 1959) have reported on magnetic-susceptibility measurements which they carried out on dilute xanthine oxidase solutions. They concluded that their data suggested the presence of free radicals outside as well as inside the enzyme; further work will be needed to establish the relationship between their work and that presented above.

SUMMARY

1. Xanthine oxidase solutions have been examined in the frozen state by means of electron-spin resonance.

2. 'Active' and 'inactive' samples of xanthine oxidase were used and measurements were made both on the resting enzyme and after treatment with either xanthine or sodium dithionite.

3. Electron-spin resonance signals consisted of two separate peaks. Under various conditions these peaks could be obtained alone or in combination with each other.

4. It is concluded that the iron of the enzyme is always in the ferrous state, and that the molybdenum is reduced and free radicals are formed when the substrate is added to the active enzyme.

We are grateful to Dr A. Ehrenberg for carrying out the susceptibility measurements and to him and to Dr P. Handler for allowing us to quote from unpublished work. We wish to thank Professor A. Tiselius, F.R.S., and Professor K. Siegbahn for their interest in the work which was supported by the Rockefeller Foundation and Swedish Natural Science Research Council. One of us (R.C.B.) also thanks Professor A. Haddow, F.R.S., and Professor F. Bergel, F.R.S., for their interest, the Institute of Cancer Research: Royal Cancer Hospital, London, for travel and maintenance grants and the Biochemical Institute, Uppsala, for laboratory facilities. Preliminary details of this work were presented at a meeting of the Biochemical Society on 23 January 1959.

REFERENCES

Adams, M., Blois, M. S., jun. & Sands, R. H. (1958). *J. chem. Phys.* **28**, 774.

Avis, P. G., Bergel, F. & Bray, R. C. (1955). *J. chem. Soc.* p. 1100.

Avis, P. G., Bergel, F. & Bray, R. C. (1956). *J. chem. Soc.* p. 1219.

Avis, P. G., Bergel, F., Bray, R. C., James, D. W. F. & Shooter, K. V. (1956). *J. chem. Soc.* p. 1212.

Avis, P. G., Bergel, F., Bray, R. C. & Shooter, K. V. (1956). In *Inorganic Nitrogen Metabolism*, p. 552. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
Beinert, H. (1956). *Biochim. biophys. Acta*, **20**, 588.
Beinert, H. (1957). *J. biol. Chem.* **225**, 465.
Bergel, F. & Bray, R. C. (1958). *Symp. biochem. Soc.* **15**, 64.
Bergel, F. & Bray, R. C. (1959). *Biochem. J.* **73**, 182.
Bernheim, F. & Dixon, M. (1928). *Biochem. J.* **22**, 113.
Clark, L. J. & Axley, J. H. (1955). *Analyt. Chem.* **27**, 2000.
Commoner, B. & Lippincott, B. B. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 1110.
Commoner, B., Lippincott, B. B. & Passonneau, J. V. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 1099.
De Renzo, E. C., Heytler, P. G. & Kaleita, E. (1954). *Arch. Biochem. Biophys.* **49**, 242.
Ehrenberg, A. & Ludwig, G. D. (1958). *Science*, **127**, 1177.
Flodin, P. & Kupke, D. W. (1956). *Biochim. biophys. Acta*, **21**, 368.
Fridovich, I. & Handler, P. (1958*a*). *J. biol. Chem.* **231**, 899.
Fridovich, I. & Handler, P. (1958*b*). *J. biol. Chem.* **233**, 1581.
Griffiths, J. H. E., Owen, J. & Ward, I. M. (1953). *Proc. Roy. Soc.* A, **219**, 526.
Gutfreund, H. & Sturtevant, J. M. (1959). *Biochem. J.* **73**, 1.
Ingram, D. J. E. (1958). *Free Radicals as Studied by Electron Spin Resonance*. London: Butterworths.
Isenberg, I. & Szent-Györgyi, A. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 857.
Mackler, B., Mahler, H. R. & Green, D. E. (1954). *J. biol. Chem.* **210**, 149.
Malmström, B. G., Mosbach, R. & Vänngård, T. (1959). *Nature, Lond.*, **183**, 321.
Morell, D. B. (1952). *Biochem. J.* **51**, 657.
Nicholas, D. J. D. & Stevens, H. M. (1955). *Nature, Lond.*, **176**, 1066.
Porath, J. (1954). *Acta chem. scand.* **8**, 1813.
Richert, D. A. & Westerfeld, W. W. (1954). *J. biol. Chem.* **209**, 179.
Siegel, S. M. & Weintraub, R. L. (1952). *Biochem. J.* **52**, 324.
Singer, T. P. & Massey, V. (1957). *Rec. chem. Progr.* **18**, 201.
Theorell, H. & Ehrenberg, A. (1951). *Ark. Fys.* **3**, 299.
Totter, J. R. & Comar, C. C. (1956). In *Inorganic Nitrogen Metabolism*, p. 513. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
Weissman, S. I. & Cohn, M. (1957). *J. chem. Phys.* **27**, 1440.
Wertz, J. E. (1955). *Chem. Rev.* **55**, 829.
Westerfeld, W. W., Richert, D. A. & Higgins, E. S. (1956). In *Inorganic Nitrogen Metabolism*, p. 492. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.

Metabolism of Xylose by the Lens

2. RAT LENS *IN VIVO* AND *IN VITRO**

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When a diet containing 35% of D-xylose is fed to a weanling rat, complete opacity of the lens (cataract) ensues. The relevant literature has been summarized earlier (van Heyningen, 1958).

Because the young rat lens is very small, the calf lens was used for a preliminary investigation of the metabolism of xylose by the lens *in vitro* (van Heyningen, 1958). In the present work, the metabolism of xylose *in vivo* and *in vitro* by the lens of the weanling rat was investigated. The cataract, which closely resembles that due to feeding with galactose or experimental diabetes, does not appear in its initial stages to be due to precipitation of the proteins of the lens, but to swelling of the outermost lens fibres (von Sallmann, Caravaggio, Grimes & Collins, 1958; Friedenwald & Rytel, 1955).

EXPERIMENTAL

Methods

Diet and examination of eyes. In feeding experiments weanling Wistar albino rats 22–24 days old were used, except in one experiment where the rats were 14 weeks old. One or more litters of the same age were divided into two groups: one group was given a xylose-containing diet and the other group an identical diet except that glucose was substituted for xylose. The diet (Darby & Day, 1939) consisted of glucose or xylose 35%, crude casein 18%, cornstarch 26%, dried baker's yeast 10%, butter 6%, salts 3% and cod-liver oil 2%. On a few occasions, indicated in the text, 25% of xylose and 10% of glucose were substituted for 35% of xylose. Examination of the eye by ophthalmoscope or slit lamp showed early signs of cataract in the xylose-fed rats after 5–6 days on the diet. After 7–8 days cataract was usually well advanced and visible to the naked eye. The animals were killed with ether, usually after 1–5 days on the diet, and the tissues removed immediately for analysis.

Preparation of tissue extracts. The lens was removed from the eye, still in the animal, by cutting the cornea with a sharp scalpel and then applying gentle pressure to the eyeball. Each lens weighed 14–18 mg. The lenses from a group of xylose-fed or glucose-fed rats were pooled, weighed and ground in ten times their weight of 5% (w/v) trichloroacetic acid; the protein precipitate was removed by centrifuging at 0° at 10 000 *g* for 20 min. The trichloroacetic acid extract was first treated with methyldioctylamine by the method of Hughes & Williamson (1951) to remove

* Part 1: van Heyningen (1958).

trichloroacetic acid. The volume of the extract was then measured so that the weight of fresh tissue it contained could be calculated. It was then taken to dryness in a desiccator. The dried extract was dissolved in a known volume of water (1–5 mg. fresh tissue/μl. of water) and stored at $-10°$. Extracts of other tissues, when required, were prepared in the same way. Aqueous humour, when required, was obtained from the eye before removal of the lens by pushing a fine capillary gently through the cornea. The aqueous humour ran into the pipette and was blown into a tared tube. This procedure was usually, but not always, successful. Between 5 and 10 mg. of aqueous humour per eye was obtained and samples from a group of animals were pooled and stored at $-10°$.

Blood, when required, was withdrawn directly into a tube containing 10% (w/v) trichloroacetic acid. When plasma was needed heparin was used as an anticoagulant. The heparinized blood was centrifuged, the plasma removed and mixed with an equal volume of 10% (w/v) trichloroacetic acid, and the protein precipitate removed by centrifuging at 0° at 10 000 *g* for 20 min. The trichloroacetic acid extract of blood or plasma was treated with methyldioctylamine (Hughes & Williamson, 1951) and then, if amino acids were to be measured, it was washed on to a column of Amberlite IR 120 (H^+); the amino acids were eluted from the column with aq. 2N-NH_3 soln. and the eluate was taken to dryness *in vacuo*. The dried extract was dissolved in a known volume of water and stored at $-10°$. Blood in which glucose and xylose were to be estimated was treated with $Ba(OH)_2$ and $ZnSO_4$ by the method of Somogyi (1945).

Preparation of lens extracts for measurement of enzyme activity in the absorption spectrophotometer. Rat lenses were ground in a mortar with five times their weight of deionized water, and the dispersion was dialysed overnight at 4° against deionized water. The dialysed dispersion was brought to pH 7·4 with dilute aq. NH_3 soln. with a glass electrode and the insoluble part was removed by centrifuging. The lens of the adult rat was used for the preparations of these extracts usually. Extracts of calf lens were made in the same way, except that only twice their weight of water was used to make the dispersion. Absorption measurements were subsequently made as described in Table 3 with a Unicam SP. 500 spectrophotometer.

Paper chromatography. Descending chromatography on Whatman no. 1 paper was used. The solvents used were: phenol–water (100:28, w/v; for the separation of amino acids, 0·3 ml. of aq. 3% NH_3 soln. was added to each 50 ml., and 6 ml. of aq. 3% NH_3 soln. in 200 ml. of water was put in the bottom of the tank); benzene–butan-1-ol–pyridine–water (1:5:3:3), upper phase (Hough, 1950); butanol–acetic acid–water (40:9:20; Waley, 1956); propan-1-ol–ethyl acetate–water (7:1:2; Albon & Gross, 1950).

For two-dimensional chromatograms a Multi-sheet Frame tank was used (Shandon Scientific Co.); the first solvent was butanol–acetic acid–water and the second phenol–aq. NH_3 soln.

Paper electrophoresis. Horizontal strips of Whatman no. 3MM paper between sheets of polythene and glass were used (Kunkel & Tiselius, 1951). The potential difference along the paper was about 10 v/cm. The method of Frahn & Mills (1956) was used for paper electrophoresis of sugar alcohols in 0·1M-sodium tetraborate, pH 9·2. A spot of phenol red (0·04%) was applied at the origin to follow the course of the electrophoresis visually; it migrates just ahead of dulcitol, the fastest moving of the sugar alcohols examined. For electrophoresis at pH 4·0, pyridine–acetic acid buffer was used (50 ml. of acetic acid, 15 ml. of pyridine and 2·5 l. of water; Grassmann, Hannig & Plöckl, 1955).

Detection and estimation of substances after paper chromatography. The $AgNO_3$–NaOH method of Trevelyan, Proctor & Harrison (1950) was used to detect carbohydrates which react under these conditions. The sensitivity of this reaction was found to be greatly reduced in the presence of sodium tetraborate, and after paper electrophoresis in a solution of this substance the method of Metzenburg & Mitchell (1954) was used. Aniline phthalate was used to detect aldoses (Partridge, 1949). Carbohydrates were estimated on paper by comparing the size of the spot produced with $AgNO_3$–NaOH with spots produced by a range of known amounts of the compound developed on the same sheet of paper.

Amino compounds were estimated quantitatively on paper by the method of Peart (1956), and compounds containing phosphorus were visualized and estimated as described previously (van Heyningen & Pirie, 1958).

Other estimations. Reduced glutathione (GSH) was estimated colorimetrically by the method of Grunert & Phillips (1951) and total glutathione chromatographically by the method of Waley (1957) or Peart (1956).

Oxidized glutathione (GSSG) in lens extracts was estimated enzymically with GSSG reductase prepared from yeast by the first three steps of the purification procedure of Racker (1955). Two rat lenses were ground with 2·5 ml. of 0·1M-veronal–acetate buffer, pH 7·2, and the dispersion was centrifuged. GSSG in 2·4 ml. of the extract was estimated by the addition of glutathione reductase (0·01 ml.) and reduced triphosphopyridine nucleotide (0·25 μmole) in a final volume of 3 ml. Decrease of absorption was measured at 340 mμ. The reduction of 0·1 μmole of GSSG took about 10 min. to go to completion.

Keto sugars were estimated by the method of Dische & Borenfreund (1951), pyruvic acid by the method of Friedemann & Haugen (1943), lactic acid by the method of Barker & Summerson (1941) and amino compounds by the method of Rosen (1956). Glucose was estimated by the method of Nelson (1944). To measure glucose in the presence of xylose, $Ba(OH)_2$–$ZnSO_4$ extracts were analysed before and after removal of glucose by yeast fermentation (King & Wootton, 1956). Standards for glucose and xylose were included. The values for xylose were checked by the method of Barrenscheen & Peham (1941).

Materials

D-Glucose, D-xylose, D-fructose and D-galactose were obtained from British Drug Houses Ltd. and diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) from Sigma Chemical Co.

For enzyme experiments the D-xylose was recrystallized from ethanol. Arabitol was kindly given by Dr O. Touster, D-xylulose by Dr J. S. Fewster and glutathione reductase by Dr A. Pirie. Xylitol was made by the method of Hollmann & Touster (1957), except that freshly prepared Raney nickel catalyst was used. Reduced DPN (DPNH) was made by the method of Slater (1953) and reduced TPN (TPNH) by the method of Evans & Nason (1953).

Table 1. *Glucose and xylose in whole blood and in the aqueous humour of weanling rats fed for 5 days on a diet containing 35 % of glucose or 35 % of xylose*

Expt. no.	Fluid analysed	Sugar fed	No. of animals	Glucose (mg./ml.)	Xylose (mg./ml.)
1	Blood	Glucose	3	0·83	0
		Xylose	3	1·00	0·48
2	Blood	Glucose	3	0·70	0
		Xylose	3	0·70	0·39
3	Blood	Glucose	2	0·88	0
		Xylose	2	0·89	0·49
4	Blood	Glucose	2	0·83	0
		Xylose	2	0·89	0·54
5	Blood	Xylose	3	1·15	0·87
6	Aqueous humour	Xylose	2	0·77	0·88
7	Aqueous humour	Xylose	8	0·60	0·64

Table 2. *R_F values and distances of various sugars and polyols in various chromatographic solvents*

Solvents: (1) Phenol–water (100:28, w/v); (2) benzene–butan-1-ol–pyridine–water (1:5:3:3); (3) butanol–acetic acid–water (40:9:20); (4) propan-1-ol–ethyl acetate–water (7:1:2).

	R_F			
Solvent ...	(1)	(2)	(3)	(4)
Xylose	0·46	0·43	0·32	—
Glucose	0·40	0·32	0·24	—
Xylulose	0·61	0·54	0·39	—
Sorbitol	0·48	0·29	0·26	—
Xylitol	0·61	0·35	0·31	—

	Distance travelled (cm.)			
Solvent ...		(2)		(4)
Sorbitol	—	13·1	—	13·9
Xylitol	—	15·7	—	16·3
Arabitol	—	17·4	—	17·6
Ribitol	—	18·0	—	17·6

RESULTS

Glucose and xylose in the blood

Table 1 gives the levels of sugars in the blood of rats fed for 5 days on the diet with 35 % of xylose or 35 % of glucose and in the aqueous humour of xylose-fed rats. Xylose has been shown to penetrate readily from the blood into the aqueous humour (Harris & Gehrsitz, 1949). Chromatographic analysis also showed that the concentration of glucose was approximately the same in the aqueous humour of xylose-fed rats and glucose-fed rats.

Carbohydrate compounds formed in the rat lens from xylose

Extracts of the lens of rats fed on a diet of 35 % of xylose or 35 % of glucose (hereafter to be referred to as 'xylose-lens' and 'glucose-lens extracts') were compared by paper chromatography. The papers were treated with $AgNO_3$–NaOH (Trevelyan *et al.* 1950). It was found that after 5 days on the diet both the xylose-lens and the glucose-lens extracts yielded faint spots of equal strength in the position of inositol and usually faint spots in the position of glucose. In addition the xylose-lens extracts showed spots corresponding to three other carbohydrate compounds, a very strong one which is almost certainly xylitol, a much weaker one corresponding in position to sorbitol and a very faint one in the position of xylose. There was no trace of xylonic acid, the oxidation product of xylose found in the calf lens after incubation in a medium containing xylose (van Heyningen, 1958). The R_F values, or distances moved, of xylose, glucose and a number of related compounds are given in Table 2. Sorbitol was identified only by its R_F in various solvents (Table 2), by the slow appearance of the $AgNO_3$–NaOH spot, and by the fact that it did not migrate when subjected to electrophoresis on paper at pH 4·0, but migrated at the same rate as an authentic sample in sodium tetraborate at pH 9·2. These same criteria were also used for the identification of xylitol; further evidence for the identity of xylitol will be presented below. By running mixed spots in two solvents with the authentic compounds it was shown to be neither of the other two pentatols, arabitol and ribitol (Table 2).

Xylitol was often found in the xylose-lens extracts after the rats had been 1 or 2 days on the diet, at a concentration of about 2 mg. of xylitol/g. of lens. With one exception it always reached at least this concentration after 5 or 6 days and was usually at about 4 mg./g. After 2 days the sorbitol spot was very faint but after 5 days sorbitol was about one-tenth as concentrated as the xylitol, as judged by the strength of the chromatographic spots. In one experiment, where a weanling rat was fed on a diet with 25 % of xylose for 25 days, the lens extract contained xylitol and sorbitol in

approximately equal amounts. No sorbitol was ever seen in the lens of the glucose-fed rat, but chromatograms in phenol–water of extracts of calf lens incubated in a medium containing glucose (5 mg./ml.) show spots in the position of sorbitol.

To see if xylitol and sorbitol also accumulate in the lens of the adult rat, which is not susceptible to xylose-cataract (Booth, Wilson & DeEds, 1953), a litter of three 14-weeks-old rats was fed on a diet with 35 % of xylose and killed after 2, 4 and 13 days on the diet. There was no sign of cataract in any of the lenses. After 2 days the lenses contained a trace of both polyols; after 4 days xylitol had reached a concentration of 0·33 mg./g. and sorbitol 1·0 mg./g. After 13 days xylitol was at a concentration of 1·5 mg./g. and sorbitol 2·0 mg./g. There was no evidence of the presence of xylose or glucose in these lenses.

Incubation of rat lens in a medium containing xylose (van Heyningen, 1958) resulted in the formation of a high concentration of xylitol; no xylonic acid was found. In this experiment *in vitro* free xylose was also found in the lens at a high concentration, whereas *in vivo* it is found in the lens only in traces and is sometimes not detectable. However, the concentration of xylose (5 mg./ml.) in the incubation medium in the experiment *in vitro* was six to eight times as high as in the aqueous humour of the xylose-fed rat.

Extracts of calf lens, incubated in the medium containing xylose (van Heyningen, 1958), were re-examined and it was found that in addition to the high concentration of xylonic acid formed, a small amount of xylitol was also present. This was missed (van Heyningen, 1958) partly because it was mistaken for xylonic acid lactone, which has the same R_F as xylitol in phenol–water, and partly because it was in the 'barium-soluble ethanol-soluble' fraction which was analysed for xylulose and phosphates but not for $AgNO_3$–NaOH-reacting substances. Thus the rat lens *in vivo* and *in vitro* makes only the reduction product xylitol, whereas the calf lens *in vitro* makes a little of this but mostly the oxidation product xylonic acid. Rabbit lens incubated *in vitro* under the same conditions was shown to make xylitol with a trace of xylonic acid.

Enzymic oxidation of xylose by extracts of rat lens

In experiments similar to those with calf-lens extracts (van Heyningen, 1958) it was shown that rat lens contains an enzyme system which reduces DPN in the presence of xylose at pH 9·0. This system was not examined any further since xylonic acid, the product of the reaction, is not formed by the intact rat lens.

Enzymic reduction of xylose, glucose, galactose and fructose by extracts of rat and calf lens

It was found that TPNH is oxidized by rat lens in the presence of xylose, galactose or glucose, but not fructose, whereas DPNH is oxidized in the presence of fructose, but not of xylose, galactose or glucose. Calf lens also contained similar enzymes but they were usually less active (Table 3).

Sugars are reduced to the corresponding sugar alcohols in the presence of polyol dehydrogenases and reduced coenzyme (McCorkindale & Edson, 1954; Hers, 1957). Thus xylitol and sorbitol are the expected products of the reaction in which TPNH is oxidized by lens extracts in the presence of xylose and glucose respectively. The formation of xylitol by an extract of rat lens in the presence of xylose and TPNH was shown as follows. Dialysed extract of rat lens (1 ml.) was incubated at 37° with TPNH (approx. 0·7 μmole), 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7·4 (10 μmoles), and xylose (6·7 μmoles) in a final volume of 3 ml. After incubation for 1 and 2 hr. more TPNH (0·7 μmole each time) was added, and after incubation for 3 hr. the reaction was stopped by the addition of 3 ml. of 10 % (w/v) trichloroacetic acid.

The extract was treated with methyldioctylamine (Hughes & Williamson, 1951), passed through a column of Amberlite IR 120 (H^+), neutralized with aq. NH_3 soln. and, after being taken to dryness *in vacuo*, redissolved in 0·1 ml. of water. Chromatography in phenol–water of 5 μl. of this solution showed, in addition to a large xylose spot, a spot in the position of xylitol, which was not present in the mixture before incubation.

Table 3. *Oxidation of reduced tri- and reduced diphosphopyridine nucleotide by extracts of dialysed rat and calf lens in the presence of various sugars*

The reaction mixture contained dialysed lens extract at pH 7·4, sugar (670 μmoles of xylose; 560 μmoles of the others), tris buffer, pH 7·4 (100 μmoles) and DPNH or TPNH (approx. 0·22 μmole) in a volume of 3 ml. Decrease in absorption at 340 mμ during 5 min. (ΔE) was measured.

Animal	Coenzyme	Sugar	ΔE/g. fresh wt. of lens
Rat	DPNH	Xylose	0
		Glucose	0
		Galactose	0
		Fructose	1·37
Rat	TPNH	Xylose	1·00
		Glucose	0·72
		Galactose	1·13
		Fructose	0
Calf	TPNH	Xylose	0·16
Cow	TPNH	Xylose	0·10
	DPNH	Fructose	0·49

Search for evidence of formation of D-*xylulose*

The results of experiments shown in Table 3 agree with the general finding that enzymes reducing aldoses use TPNH and those reducing ketoses use DPNH (see de Duve & Hers, 1957). There is one exception to this finding, however, namely an enzyme in liver mitochondria which reduces L-xylulose to xylitol, with TPNH as co-enzyme (Hollmann & Touster, 1957). In view of this, evidence for the formation by rat lens of xylulose from xylose was sought. An isomerase which converts D-xylose into D-xylulose has been found in bacteria (Kline & Baron, 1957; Litthauer, Volcani & Bergmann, 1955; Hochster & Watson, 1954) but not in animal tissues.

The method of Hochster & Watson (1954) was used, in which any xylulose which might be formed by the reaction of lens with xylose in the absence of TPNH is trapped by borate buffer, thus forcing the reaction in the direction of the formation of xylulose, and preventing it from being metabolized further. D-Xylose (1 μmole), in 0·15 ml. of 0·2 M-borate buffer, pH 8·0, was incubated at 37° with 0·15 ml. of 1 in 4 dilution of dialysed rat-lens dispersion. The lens of the weanling rat was used in this experiment. Controls without xylose and without lens were incubated at the same time, and 0·1 ml. samples were pipetted into 0·5 ml. of 0·1 N-HCl at 0 and 4 hr. Analysis of the extracts for keto sugars showed no formation of xylulose from xylose by lens.

Enzymic oxidation of xylitol by extracts of rat lens

As expected, it was found that xylitol in the presence of dialysed rat lens and TPN was oxidized to the aldose sugar xylose whereas in the presence of DPN the keto sugar xylulose was formed.

Xylitol and diphosphopyridine nucleotide. The reaction with DPN was coupled with lactic acid dehydrogenase and pyruvate, in order to favour the formation of xylulose. Dialysed rat-lens extract (0·75 ml.) was incubated with xylitol (48 μmoles), sodium pyruvate (50 μmoles) and DPN (1 μmole) in a final volume of 1·3 ml. at pH 7·5; rat lens contains an active lactic dehydrogenase. A control without DPN was prepared at the same time; after 6 hr. at 37° 2 ml. of 10 % (w/v) trichloroacetic acid was added to each tube. The extracts were treated with methyldioctylamine, passed through a column of Amberlite IR 120 (H^+), neutralized with aq. NH_3 soln., taken to dryness *in vacuo* and redissolved in a small volume of water. Chromatography, followed by treatment of the paper with $AgNO_3$–NaOH, showed a spot in the position of xylulose, which was not present when DPN was absent from the incubation mixture. This was confirmed by the finding that keto sugar was formed in the presence but not in the absence of DPN.

Xylitol and triphosphopyridine nucleotide. Dialysed rat-lens extract (1·5 ml.) and 0·5 ml. of 0·1 M-tris buffer, pH 8·0, were incubated at 37° with TPN (3 μmoles) and xylitol (20 μmoles) in a final volume of 3 ml. The rate of formation of TPNH was followed spectrometrically. After 6 hr., when about 0·5 μmole of TPNH had been formed and the reaction had nearly stopped, trichloroacetic acid was added to the reaction mixture and to the control, which was identical except for the omission of TPN. The extracts were prepared for chromatography as described in the experiment above, and it was shown by means of the aniline phthalate spray of Partridge (1949) that the extracts incubated with TPN contained a reducing sugar with the same R_F as xylose and which was not present in the control.

Search for xylitol in tissue other than lens

The only animal tissues (apart from the lens) in which dehydrogenases reducing D-xylose to xylitol have been found are the seminal vesicles and placenta of the sheep (Hers, 1956, 1957).

In order to see if xylitol accumulates in tissues of the weanling rat other than the lens, trichloroacetic acid extracts of blood, kidney, lung, liver, heart, muscle, retina, testis and spleen were made from a rat fed with 35 % of xylose for 5 days, and analysed chromatographically. No xylitol (or xylonic acid) was detected in an amount of extract equivalent to 15 mg. of wet tissue. Since 5 μg. of xylitol is readily detected, the concentration in these tissues was less than 0·33 mg./g. An extract from 2 mg. of lens tissue gave a large spot corresponding to xylitol and an extract of 5 mg. of aqueous humour gave a faint spot of xylitol, presumably derived from the lens. No xylitol was detected in the seminal vesicles of a 16-weeks-old rat fed with a diet of 35 % of xylose for 2 weeks.

Reversibility of xylose cataract

Booth *et al.* (1953) showed that regression of lens opacity occurred with time, even though the xylose diet was continued. This finding was confirmed. After 14 days on a diet with 25 % of xylose a weanling rat had almost complete cataract in both lenses. After another 12 days regression had begun and when the rat had eaten the diet for 67 days the cataract had almost disappeared, and clear fibres had formed outside the remaining opacities. The rat was killed and the lenses were found to contain a high concentration of xylitol, about 1 mg./g. of lens, and a lower concentration of sorbitol.

Glutathione in the lens of the xylose-fed rat

In many types of experimental cataract, a decreased level of GSH within the lens precedes the appearance of the opacity; GSSG is not formed (see

Pirie & van Heyningen, 1956). Table 4 shows the difference in concentration of reduced and total glutathione in xylose-lens and glucose-lens extracts after the rats had been fed for 1–7 days on the experimental diet. Owing to the small amount of tissue used for the estimation of GSH (one or two lenses of 14–18 mg. weight) small differences between the xylose-lens and glucose-lens are not significant. Table 4 shows that, when xylose is fed to rats, the level of GSH in the lens probably begins to diminish at about the same time as the first appearance of opacities. No GSSG was found in the lens of the normal rat or of the rat fed on the xylose diet for 2, 4 or 7 days.

Search for other changes in rat lens as a result of feeding with xylose

Groups of rats fed on a diet containing 35% of xylose, and their litter mates fed on a diet containing 35% of glucose, were killed at various intervals after the start of the experimental diets. The concentration of lactic acid, total P, inorganic P (P_i), P contained in compounds hydrolysed to P_i in 10 min. in N-HCl at 100°, and P contained in compounds not hydrolysed to P_i under these conditions, were compared in the two groups of lenses. Xylitol was usually present at a high concentration after 1 day on the xylose diet, although in one experiment it was at a low level after 5 days. On one occasion adenosine triphosphate (ATP) was kindly measured by Dr G. Born [by the specific firefly method (Strehler & Totter, 1954)] in the lenses of rats fed for 3 days on the diet and there was no difference between the two groups. There was also no significant difference in the concentration of the sum of the two compounds phosphorylcholine and glycerophosphorylcholine on the two occasions they were measured by their phosphorus content in chromatographic spots.

The concentrations of the compounds in the lens were: lactic acid 1·0–1·2 mg./g. of lens; total P, inorganic P, organic P hydrolysed to P_i in N-HCl at 100° for 10 min., and organic P not hydrolysed under these conditions, 0·81, 0·10, 0·24 and 0·47 mg./g. of lens respectively. ATP in the lens, measured by the firefly method, was at a concentration of 2·85 mM. This is equivalent to 0·18 mg. of 'easily hydrolysible' P/g. of lens and therefore accounts for most of the measured 'easily hydrolysible' P. Phosphorylcholine and glycerophosphorylcholine together accounted for 0·16 mg. of P/g. of lens.

Mandel, Schmit & Quaranta (1956) found a decreased level of pyruvic acid in the lens of the rat made diabetic with alloxan. Table 5 gives the value for pyruvic acid in the lens and blood of xylose- and glucose-fed rats. Very often there was a decrease of pyruvic acid in the xylose-lens but this was not a consistent finding. On one of the two occasions on which the level in the blood was also measured it was raised in the xylose-fed rat and also in the lens. On the other occasion it was decreased in the blood and unaltered in the lens. Probably lens pyruvic acid at least partly reflects the level in blood and aqueous humour which in turn are dependent on many other variables.

Table 4. *Glutathione content of the lens of the xylose-fed rat relative to that of the glucose-fed rat*

Weanling rats were fed on a diet containing 35% of xylose or 35% of glucose for the stated number of days. For estimations of reduced and total glutathione, see Methods section. Concentration of GSH in the lens of the rat fed with glucose was 1·5–2·2 mg./g. of tissue.

Period on diet (days)	Concn. of glutathione in lens of xylose-fed rat expressed as percentage of that in lens of glucose-fed rat: Reduced glutathione	Total
1	110	—
1	—	89
2	111	—
2	—	89
2	—	100
2	—	111
3	117	—
3	—	78
3	—	100
4	81	—
4	—	89
5*	90	—
5*	—	53
6*	87	—
6†	34	—
7†	30	—

* Early stages of cataract.
† Lens severely cataractous.

Table 5. *Pyruvic acid content of the lens and blood of the xylose-fed rat, compared with that of the glucose-fed rat*

Weanling rats were fed a diet of 35% of xylose or 35% of glucose for the stated number of days. Pyruvic acid was estimated by the method of Friedemann & Haugen (1943).

Period on diet (days)	Concn. of pyruvic acid in lens or blood of xylose-fed rat expressed as percentage of that in glucose-fed rat: Lens	Blood
1	88	—
2	52	—
2	102	82
2	111	—
2	65	—
3	56	—
3	120	164
4	76	—
4	79	—

The concentration of pyruvic acid in the rat lens was 0·03–0·04 mg./g. of lens, a value similar to that found by Mandel *et al.* (1956).

Free amino acids in the lens

Comparison between the lens of xylose-fed and glucose-fed rats. By examination of extracts of xylose-lens and glucose-lens on two-way chromatograms it was possible to compare the concentrations of a number of the amino acids, by the method of Peart (1956). In lens extracts which had been taken to dryness and redissolved in water, all the glutathione had become oxidized and therefore ran as a single spot near the origin and well separated from all the amino acids. Table 6 shows that after 3 days on the diet the concentrations of some of the amino acids, and of glutathione, were considerably reduced. In one experiment, in which the rats were killed after only 1 day on the experimental diet, there was 40 % less free alanine in the xylose-lens than in the glucose-lens, whereas there appeared to be no difference in the concentration of other amino acids or of total glutathione. Amino acids were identified only by their relative positions on the chromatograms, compared with the positions of known amino acids run at the same time on separate chromatograms.

Amino acids in blood plasma of xylose-fed and glucose-fed rats. To find whether the changes shown in Table 6 were a reflexion of similar changes in the

Table 6. *Free amino acids in the lens of the xylose-fed and glucose-fed rat*

Weanling rats were fed on a diet of 35 % of xylose or 35 % of glucose for 3 days. Free amino acids in 125 mg. of lens were compared on two-dimensional chromatograms (see Methods section) sprayed with the ninhydrin reagent of Peart (1956). The coloured spots were cut out, extracted with 0·7 or 3·5 ml. of 70 % (v/v) acetone (Peart, 1956) according to their intensity, and the extinctions of the coloured solutions at 580 mμ were measured in the Unicam spectrophotometer, in cells of 1 cm. length and capacity 0·5 ml. Extinctions of extracts made in a volume of 3·5 ml. are multiplied by five to make them comparable with those made in 0·7 ml.

Amino acid	*E*		Concn. of amino acid in lens of xylose-fed rat expressed as percentage of that in the glucose-fed rat
	Glucose-lens	Xylose-lens	
Glutathione	2·62	2·34	89
Alanine	2·35	0·83	35
Glutamic acid	2·21	1·40	63
Taurine and serine	1·97	1·57	80
Lysine and histidine	1·54	1·46	97
Threonine and glycine	0·70	0·39	55
Leucine and *iso*leucine	0·66	0·59	90
α-Aminobutyric acid	0·38	0·17	31
Glutamine	0·36	0·24	68
Methionine and valine	0·34	0·38	111
Aspartic acid	0·21	0·11	53

Table 7. *Difference between concentration of free amino acids in the blood plasma and lens of the xylose-fed and the glucose-fed rat*

Weanling rats were fed on a diet of 35 % of xylose or 35 % of glucose for 5 days. Free amino acids in 25 mg. of lens or 125 mg. of plasma were measured on two-dimensional chromatograms (see Methods section) and sprayed with the ninhydrin reagent of Peart (1956). The coloured spots were cut out, eluted with 0·7 ml. of 70 % (v/v) acetone and the extinctions measured at 580 mμ in the spectrophotometer, in cells of 1 cm. length and 0·5 ml. capacity. Values for the lens are all multiplied by five to make them comparable with those for plasma. Thus all the extinctions refer to 125 mg. of tissue.

Amino acid	*E*				Concn. of amino acid in tissue of xylose-fed rat expressed as percentage of that in glucose-fed rat	
	Glucose-fed rat		Xylose-fed rat			
	Lens	Plasma	Lens	Plasma	Lens	Plasma
Glutathione	5·07	—	2·67	—	53	—
Alanine	2·75	0·74	0·32	0·45	12	61
Glutamic acid	3·81	0·64	1·09	0·63	29	98
Taurine	4·60	—	2·29	—	50	—
Serine	—	0·35	—	0·38	—	109
Lysine and histidine	4·38	0·44	1·94	0·33	44	75
Threonine and glycine	2·37	0·62	0·88	0·49	37	78
α-Aminobutyric acid	0·81	—	0·92	—	113	—
Glutamine	—	0·45	—	0·32	—	72
Methionine and valine	—	0·10	—	0·09	—	88

blood, and therefore presumably in the aqueous humour, the concentrations of some of the amino acids of the blood plasma of xylose-fed and glucose-fed rats were compared by the method of Peart (1956) after 2–5 days on the diet. Table 7 shows that the decreases of amino acids in the lens are at least partly a reflexion of similar changes in the blood plasma, although the changes in the lens are greater than those in the plasma.

There were decreases in the amounts of other amino acids, which were at too low a concentration, or not sufficiently separated, for a quantitative estimation to be made. Comparisons of the amounts of free amino acids in the whole blood showed decreases in the xylose-fed rat, similar to those found in blood plasma.

There was a 31 % reduction in the total amount of amino compounds in the aqueous humour of the xylose-fed rat compared with the amount in the glucose-fed control; but there was not enough aqueous humour for chromatography.

Apart from the fact that most of the xylose in the diet fed to the rats receiving xylose would not be utilized (McCormick & Touster, 1957), these rats usually ate a considerably smaller weight of food than did the glucose-fed controls.

Effect of xylose in the diet on free amino acids in blood plasma and lens. To determine whether the reduction of free amino acids in the plasma was due to the presence of xylose in the diet, or merely to the lowered food intake of the xylose-fed rats compared with the glucose-fed controls, the following experiment was carried out. A litter of nine rats, aged 23 days, was divided into three groups. The first group was fed for 5 days on the diet containing 35 % of glucose *ad lib.* The second group was fed for 5 days on the diet containing 35 % of xylose, also *ad lib.* The third group was given the weight of food eaten by the second group, less the xylose; thus it was given 65 % of the weight of the food eaten the day before by this group, i.e. it had the same amount of food to eat as the xylose-fed rats, less the xylose. This 'sugar-free' diet was necessarily started 1 day later than the other two, but was continued for 5 days before the animals in this group were killed.

The same amino acids were measured. Whereas the glucose-fed rats gained weight steadily, both xylose-fed rats and those on the 'sugar-free' diet lost weight, the former more rapidly than the latter. (In experiments of longer duration, rats fed on the xylose diet gained weight steadily though more slowly than the glucose-fed rats.) It appeared that xylose was not itself responsible for the lowered level of amino acids in the plasma, since in the first of three experiments there was as great a decrease of amino acids in the plasma of the rats on the 'sugar-free' diet as in those fed with xylose. In two other experiments only amino acids in the plasma of the xylose-fed rats were affected, those in the rats on the 'sugar-free' diet being the same as those of the glucose-fed controls. However, in one of these two experiments the lenses were also analysed, and it was found that although the amino acids were unchanged in the plasma of the rats on the 'sugar-free' diet those of the lens had decreased to 60–70 % of their level in the lenses of the glucose-fed controls. The amino acids in the lenses of the xylose-fed rats had decreased even more, to 12–53 % of the value in the glucose-fed controls.

DISCUSSION

Evidence of identity of xylitol in the lens of the xylose-fed rat

An attempt was made to isolate the compound formed by rat lens from xylose by chromatography of xylose-lens extracts in phenol–water and elution of the appropriate area. It was not found possible, however, with the small amounts which could be collected. That the substance is almost certainly xylitol is demonstrated by the following findings.

(1) It has the same R_F as xylitol in four solvents and is thus distinguished from arabitol or ribitol (Table 2). (2) It reacts slowly with the silver nitrate–sodium hydroxide spray. (3) It has no net charge at pH 4·0, shown by paper electrophoresis. (4) It migrates at the same rate as authentic xylitol in paper electrophoresis at pH 9·2 in sodium tetraborate. (5) A compound with the same R_F as xylitol is formed from xylose by dialysed lens extracts by the oxidation of TPNH but not of DPNH. (6) Authentic xylitol is oxidized by dialysed rat-lens extract with the reduction of TPN or DPN to form compounds with the same chromatographic R_F values as xylose and xylulose respectively.

Metabolism of xylose by the lens

It has been shown that xylose is reduced by the lens in one species of animal (the rat), whereas it is oxidized, and also to a lesser extent reduced, by the lens of another species of animal (the calf). These findings apply to the rat lens *in vivo* and *in vitro* and the calf lens *in vitro*. The rabbit lens *in vitro* behaves like the rat lens, reducing xylose to xylitol. In the calf lens it appears that the action of xylose dehydrogenase, in the presence of its substrate, interferes with the glycolytic cycle by competing with triose phosphate dehydrogenase for the available DPN (van Heyningen, 1958). The rat lens, on the other hand, reduces xylose through xylitol dehydrogenase and TPNH, and by analogy one might expect competition between this system and some other system which normally uses TPNH as coenzyme. Glutathione reductase answers this

description and is present in lens (van Heyningen & Pirie, 1953), but there is no evidence that it is affected by xylose since GSSG does not accumulate and total glutathione is not diminished at the time when xylitol has reached a high concentration in the lens.

In the lens the rate of the hexokinase reaction is believed to limit the rate of glycolysis (see Pirie & van Heyningen, 1956). Xylose has been shown to inhibit hexokinase *in vitro* (Sols & Crane, 1954) but does not appear to do so in the rat lens *in vivo* since glycolysis is unaffected. If glycolysis is not interfered with it seems likely that the initial disturbance occurs in the oxidative metabolism. Only about 2·3 % of the glucose used by the rabbit lens is oxidized (Kinoshita & Wachtl, 1958), but oxygen is essential for the lens and even a small decrease of the already small uptake might be disastrous to maintenance of transparency. The fact that nearly all the carbon dioxide formed by the rabbit lens is derived from oxidation of the C-1 atom of glucose implies that the pentose phosphate pathway is predominant over the citric acid cycle (Kinoshita & Wachtl, 1958). Possibly reoxidation of TPNH produced in the two early stages of the pentose phosphate cycle is the process with which xylitol dehydrogenase competes. A cytochrome *c* reductase, with TPNH, is found in the lens of cattle and rabbit (Pirie, van Heyningen & Boag, 1953); unfortunately the amount of cytochrome *c* in the lens is so small that an investigation of the relative proportions of reduced to oxidized cytochrome *c* is not possible. On the other hand, if the rat lens contains a coenzyme transhydrogenase (Colowick, Kaplan, Neufeld & Ciotto, 1952), TPNH might be maintained in the reduced state in the presence of xylose while DPNH became oxidized. An analysis of the ratios TPNH/TPN and DPNH/DPN during the course of cataract formation might be illuminating. Furthermore, such an analysis applied to the normal lens of the calf and the rat might help to explain the finding that xylose is mostly oxidized in the former by an enzyme with DPN, and reduced in the latter by an enzyme with TPNH. There is little in the literature about the comparative concentrations of enzymes and coenzymes in the lenses of different species of animal; but these experiments suggest a great difference between some aspects of the metabolism of the calf lens and that of the rat and the rabbit.

Xylose metabolism by the intact animal

Xylose is metabolized only to a small extent by the rat (Miller & Lewis, 1932). About 1 % of injected xylose labelled with ^{14}C is probably converted into glycogen in the liver (Hiatt, 1957) and about 10 % is oxidized to carbon dioxide. In the guinea pig 75 % of injected xylose is excreted in the urine (McCormick & Touster, 1957). In man, 13·5 % of administered [^{14}C]xylose is expired as carbon dioxide: all the ^{14}C excreted is in the form of unchanged sugar, no xylonic acid or xylitol being detectable (Wyngaarden, Segal & Foley, 1957). The present work shows that the level of xylose in the blood of the rat fed on a diet containing 35 % of xylose remains low and the level of glucose does not seem to be much affected.

Metabolism of xylose and xylitol by animal tissues

The way in which xylose enters the metabolism of the mammal is not known. No isomerase converting xylose into xylulose has been found and systems which phosphorylate xylose directly appear to be lacking. Apart from the lens, only in the seminal vesicles and the placenta has an enzyme catalysing the interconversion of D-xylose and xylitol, TPN being used as coenzyme, been found (Hers, 1956, 1957). The present work shows that, of all the organs examined, only the lens accumulates xylitol but this is perhaps not surprising in view of the fact that enzymes in the liver would convert any xylitol which might be formed from xylose into D- or L-xylulose and thence by recognized metabolic pathways (McCormick & Touster, 1957). Only 25 % of the ^{14}C of labelled xylitol administered to the guinea pig is excreted in the urine; 10 % is expired as carbon dioxide and the rest is presumably incorporated into metabolic intermediates, some of it possibly in the form of glycogen (McCormick & Touster, 1957). Evidence has been given that the lens contains an enzyme which converts xylitol into xylulose in the presence of DPN. The xylulose is presumably D-xylulose, since only this isomer has been shown to be formed from xylitol by other tissues in the presence of DPN (de Duve & Hers, 1957). The equilibrium of this reaction is in favour of the formation of xylitol, but removal of D-xylulose would force the reaction in the direction of breakdown of xylitol. The fact that no xylulose has been found in the lens of the xylose-fed rat, in spite of the high concentration of xylitol, suggests that it is removed as soon as it is formed, presumably by way of xylulokinase and the pentose phosphate cycle, and the limiting reaction is that converting xylitol into D-xylulose.

Formation of sorbitol

Extracts of rat lens reduce glucose to sorbitol with TPNH as coenzyme (Table 3); possibly the enzyme is the same as that which reduces xylose and galactose. There is a gradual accumulation of sorbitol in the lens of the xylose-fed rat, whereas it was not detected in the lens of the normal weanling rat. It is difficult to see why the metabolism of the

lens of the xylose-fed rat, which is presumably deflected by reduction of xylose in the direction of oxidation of TPNH, is further deflected in this direction by the reduction of glucose, which is apparently not reduced by the lens under normal circumstances. Possibly glucose is always reduced to sorbitol by the lens, and its utilization is defective in the lens of xylose-fed rats. Both xylitol and sorbitol accumulate in the lens of the adult rat which does not develop xylose cataract, and they were also found in a lens which had almost recovered from a severe xylose cataract, so neither of these compounds can in itself be responsible for the formation of cataract. The situation seems to be that formation of xylitol and sorbitol in the fast-growing fibres of the lens in the weanling rat deranges some part of the metabolism to the extent that transparency can no longer be maintained, whereas older fibres, which are not growing so quickly, can metabolize xylose and at the same time maintain transparency.

It appears from preliminary experiments that the lens of the weanling rat fed on a diet containing 35 % of galactose accumulates dulcitol, and the lens of the rat made diabetic with alloxan accumulates sorbitol (van Heyningen, 1959).

Lowered level of free amino acids in the lens of the xylose-fed rat

The lens of the xylose-fed rat contains a lower level of free amino acids than the lens of the glucose-fed control (Tables 6 and 7). This appears to be partly a reflexion of similar changes in the blood. The loss in amino acids in the lens seems to follow a fairly regular pattern, alanine being the first to diminish, followed usually by threonine plus glycine and aspartic acid; after 5 days on the xylose diet nearly all the amino acids and also glutathione were at a lower level in the lens of the xylose-fed rat than in the lens of the glucose-fed rat. It must be stressed that the assignation of the chromatographic spots produced by the individual amino acids is only tentative, but there is no reason to doubt that most of them are correctly assigned, since they are found in general in animal tissues (Tallan, Moore & Stein, 1954; du Ruisseau, Greenstein, Winitz & Birnbaum, 1957). The relatively high concentration of α-aminobutyric acid is an exception and this may well be wrongly named.

The present work shows that a lowered food intake alone can bring about a decrease of amino acids in the lens. Cataract has never been shown to be caused by starvation but it is caused in young rats by feeding with a diet lacking in certain amino acids, in particular tryptophan (Totter & Day, 1942). The clinical appearance of this type of cataract is different from that of xylose cataract and it takes longer to develop (Buschke, 1943). The level of amino acids in the lens after feeding with a diet deficient in a single amino acid has not been examined, and the effect on the transparency of the lens of feeding with such a diet has been investigated for only a few of the individual amino acids. In the present work only the levels of those amino acids which normally occur at relatively high concentrations in the lens have been measured. A great deal more evidence is needed in order to decide whether the lowered level of amino acids in the lens as a result of xylose feeding is a primary cause of xylose cataract.

SUMMARY

1. The lens of the weanling rat fed on a diet containing 35 % of xylose for 1–7 days accumulates xylitol, which reaches a concentration of about 4 mg./g. of lens. The first signs of cataract usually appear after 5 days. No xylulose, xylonic acid or phosphorylated derivatives of xylose could be detected in the lens and very little free xylose is present.

2. Sorbitol also accumulates in the lens of the xylose-fed rat, but was not detected in the lens of glucose-fed controls.

3. Xylitol accumulates in the lens before clinical signs of cataract are present. Xylitol and sorbitol are found in the lens of the xylose-fed adult rat, which is not susceptible to xylose cataract.

4. Cataract caused by feeding with xylose was reversible and in the lens after recovery xylitol and sorbitol are found in high concentration.

5. Xylitol did not accumulate in other tissues of the xylose-fed rat, except in the aqueous humour where a small amount was found.

6. Extracts of rat lens reduce D-xylose to xylitol by means of an enzyme with reduced triphosphopyridine nucleotide as coenzyme. Glucose and galactose are also reduced by this system. Fructose is reduced by extracts of rat lens in the presence of reduced diphosphopyridine nucleotide. Xylitol is oxidized by rat lens to form xylose and xylulose in the presence of tri- and di-phosphopyridine nucleotide respectively.

7. No difference was observed in the concentration of lactic acid, inorganic phosphate, acid-labile phosphate or acid-stable phosphate between the lens of the xylose-fed rat and that of the glucose-fed control.

8. Glutathione in the lens does not decrease in concentration for the first few days of feeding with xylose, and no oxidized glutathione was found in the lens.

9. The lens and blood plasma of the xylose-fed rat contained lower amounts of free amino acids

than in the glucose-fed controls; the decreases in the lens were greater than those in the plasma.

10. The intact calf lens *in vitro*, as well as oxidizing xylose to xylonic acid (van Heyningen, 1958), also forms some of the reduction product, xylitol. The intact rat lens *in vitro* forms only xylitol from xylose.

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REFERENCES

Albon, N. & Gross, D. (1950). *Analyst*, **75**, 454.
Barker, J. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
Barrenscheen, H. K. & Peham, A. (1941). *Hoppe-Seyl. Z.* **272**, 81.
Booth, A. N., Wilson, R. H. & DeEds, F. (1953). *J. Nutr.* **49**, 347.
Buschke, W. (1943). *Arch. Ophthal., Chicago*, **30**, 735.
Colowick, S. P., Kaplan, N. O., Neufeld, E. F. & Ciotto, M. M. (1952). *J. biol. Chem.* **195**, 95.
Darby, W. J. & Day, P. L. (1939). *Proc. Soc. exp. Biol., N.Y.*, **41**, 507.
de Duve, C. & Hers, H. G. (1957). *Annu. Rev. Biochem.* **26**, 149.
Dische, Z. & Borenfreund, E. (1951). *J. biol. Chem.* **192**, 583.
du Ruisseau, J. P., Greenstein, J. P., Winitz, M. & Birnbaum, S. M. (1957). *Arch. Biochem. Biophys.* **68**, 161.
Evans, H. J. & Nason, A. (1953). *Plant Physiol.* **28**, 233.
Frahn, J. L. & Mills, J. A. (1956). *Chem. & Ind.* p. 578.
Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
Friedenwald, J. S. & Rytel, D. (1955). *Arch. Ophthal., Chicago*, **53**, 825.
Grassmann, W., Hannig, K. & Plöckl, M. (1955). *Hoppe-Seyl. Z.* **299**, 258.
Grunert, R. R. & Phillips, P. H. (1951). *Arch. Biochem. Biophys.* **30**, 217.
Harris, J. E. & Gehrsitz, L. B. (1949). *Amer. J. Ophthal.* **32**, no. 6, part 2, 167.
Hers, H. G. (1956). *Biochim. biophys. Acta*, **22**, 202.
Hers, H. G. (1957). *Le Métabolisme de Fructose*. Brussels: Éditions Arsica.
Hiatt, H. H. (1957). *J. biol. Chem.* **224**, 851.
Hochster, R. M. & Watson, R. W. (1954). *Arch. Biochem. Biophys.* **48**, 120.
Hollmann, S. & Touster, O. (1957). *J. biol. Chem.* **225**, 87.
Hough, L. (1950). *Nature, Lond.*, **165**, 400.
Hughes, D. E. & Williamson, D. H. (1951). *Biochem. J.* **48**, 487.
King, E. J. & Wootton, I. D. P. (1956). *Microanalysis in Medical Biochemistry*, 3rd ed. London: J. and A. Churchill Ltd.
Kinoshita, J. H. & Wachtl, C. (1958). *J. biol. Chem.* **233**, 5.
Kline, E. S. & Baron, L. S. (1957). *Arch. Biochem. Biophys.* **66**, 128.
Kunkel, H. G. & Tiselius, A. (1951). *J. gen. Physiol.* **35**, 89.
Litthauer, V. Z., Volcani, B. E. & Bergmann, E. D. (1955). *Biochim. biophys. Acta*, **18**, 523.
McCorkindale, J. & Edson, N. L. (1954). *Biochem. J.* **57**, 518.
McCormick, D. B. & Touster, O. (1957). *J. biol. Chem.* **229**, 451.
Mandel, P., Schmit, M. & Quaranta, C. A. (1956). *Bull. Soc. Chim. biol., Paris*, **38**, 441.
Metzenberg, R. L. & Mitchell, H. K. (1954). *J. Amer. chem. Soc.* **76**, 4187.
Miller, M. M. & Lewis, H. B. (1932). *J. biol. Chem.* **98**, 133.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
Peart, W. S. (1956). *Biochem. J.* **62**, 520.
Pirie, A. & van Heyningen, R. (1956). *Biochemistry of the Eye*. Oxford: Blackwell Scientific Publications.
Pirie, A., van Heyningen, R. & Boag, J. W. (1953). *Biochem. J.* **54**, 682.
Racker, E. (1955). *Methods in Enzymology*, vol. 2, p. 722. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Rosen, H. (1956). *Arch. Biochem. Biophys.* **67**, 10.
Slater, E. C. (1953). *Biochem. J.* **53**, 157.
Sols, A. & Crane, R. K. (1954). *J. biol. Chem.* **210**, 581.
Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.
Strehler, B. & Totter, J. R. (1954). *Methods of Biochemical Analysis*, **1**, p. 341. New York: Interscience Publishers.
Tallan, H. H., Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 927.
Totter, J. R. & Day, P. L. (1942). *J. Nutrit.* **24**, 159.
Trevelyan, W. E., Proctor, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
van Heyningen, R. (1958). *Biochem. J.* **69**, 481.
van Heyningen, R. (1959). *Nature, Lond.*, (in the Press).
van Heyningen, R. & Pirie, A. (1953). *Biochem. J.* **53**, 436.
van Heyningen, R. & Pirie, A. (1958). *Biochem. J.* **68**, 18.
von Sallmann, L., Caravaggio, L., Grimes, P. & Collins, E. M. (1958). *Arch. Ophthal., Chicago*, **59**, 55.
Waley, S. G. (1956). *Biochem. J.* **64**, 715.
Waley, S. G. (1957). *Biochem. J.* **67**, 172.
Wyngaarden, J. B., Segal, S. & Foley, J. B. (1957). *J. clin. Invest.* **36**, 1395.